A "cell-free" system to study regulation of focal adhesions and of the connected actin cytoskeleton.

Thesis

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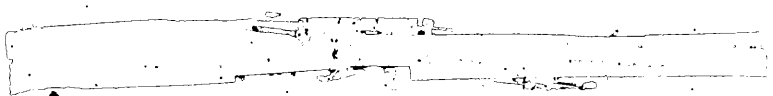
A "CELL-FREE" SYSTEM TO STUDY REGULATION OF FOCAL ADHESIONS AND OF THE CONNECTED ACTIN CYTOSKELETON

Thesis submitted in partial fulfilment of the requirements of the Open University for the degree of Doctor in Philosophy in Molecular and Cellular Biology



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DECLARATION

This Thesis has been composed by myself and has not been used in any previous application for a degree. The scanning force microscopy was performed by David Dunlap and the immunoelectron microscopy by Mario Bossi, in my Institute. All the other results presented here were obtained by myself. Chiara Albertinazzi and Stefano Cairo have helped me with some of the biochemical experiment and with the production of monoclonal antibodies. All sources of information are acknowledged by means of reference.

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ABSTRACT

The exact composition of focal adhesions and the role of single components in their assembly and regulation during dynamic adhesive processes are poorly understood. I have characterised and used a "cell-free" system consisting of ventral plasma membranes (VPMs) prepared from adherent fibroblasts, and found that this subcellular fraction preserves intact focal adhesion components and the connected actin cytoskeleton. Moreover, VPMs show an accumulation of several tyrosine phosphorylated proteins, including tyrosine phosphorylated focal adhesion kinase and paxillin. In particular, a pool of higly phosphorylated paxillin is found in focal adhesions, suggesting an important role (for) of the phosphorylated polypeptide in the mechanism of integrin-mediated adhesion.

With the aim of obtaining new important tools for my studies, I have produced and characterised monoclonal antibodies (mAbs) raised against VPM preparations.

The use of VPM preparations as a "cell-free" system has shown that changes in $[Ca^{2+}]$ can affect integrin behaviour within VPMs. I observed a correlation between integrin localisation and the functional state of the receptors, which can be reversibly modulated either by changes in free $[Ca^{2+}]$, or by function modulating anti-integrin β1 mAbs. $[Ca^{2+}]$-induced integrin redistribution is dependent on the presence of the β1 cytoplasmic domain, whereas it is independent from the presence of filamentous actin (F-actin) and focal adhesion components in this experimental system, thus implicating the uncoupling of events relevants for focal adhesion assembly under "cell-free" conditions. Moreover, reconstitution experiments show that α-actinin colocalises and redistributes with β1 receptors on VPMs depleted of actin, implicating binding of α-actinin to the receptors. Finally, I found that recruitment of exogenous actin is specifically restricted to focal adhesions under conditions in which new actin polymerisation is inhibited. These data attest the value of the system for further analysis of the molecular mechanisms regulating integrin function and focal adhesions.

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INTRODUCTION

In multicellular organisms the interactions between cells and the extracellular environment are fundamental both during embryonic development and during adulthood. In fact, the processes of controlled morphogenesis and appropriate tissue growth and repair require cells to continuously sense their relationship to the extracellular matrix (ECM) and to each other. These interactions are mediated by different families of receptors present on the surface of the cell, including growth factors receptors, cadherins, immunoglubulin superfamily receptors (NCAM, ICAM, etc...), selectins and integrins.

Integrins are the most important family of cell surface receptors that mediate attachment to and signalling from the ECM. Specific classes of integrins mediate also important cell-cell adhesive interactions. Integrin-mediated adhesive interactions are intimately involved in the regulation of many cellular functions (Fig. 1.1). During embryonic development they mediate cell migration towards the correct targets, and control the organisation of individual cells into tissues. In the adult animal, they are involved in co-stimulation and migration of lymphocytes, as well as in wound repair, bone resorption, clot retraction, and the response of cells to mechanical stress. Integrins cooperate with growth factors to promote cell proliferation, a mechanism explained, in part, by the anchorage-dependence of the G1-to-S phase transition during cell cycle progression. Adhesion is necessary also for cells to exit from the cell cycle and to differentiate. In addition, when adherent cells are released from their surrounding ECM, they forfeit survival signals and undergo apoptosis. The great effort in the last years in the understanding of the molecular basis of adhesion regulation and integrin-mediated signalling is justified also by the involvement of these receptors in several pathological situations, including tumour cell growth and metastasis, thrombosis and inflammation.

Integrin signalling leading to these different functions is initiated by the interaction of the receptors with the extracellular ligand, a mechanism that will be discussed in 1.1. Integrin affinity for the ligand is often regulated by cells, through a mechanism called "inside-out signalling", that allows cells to modify the strength of adhesion during several important processes, such as migration: the molecular basis of this phenomenon will be discussed in 1.2. Signal transduction initiated by integrins involves the interaction and

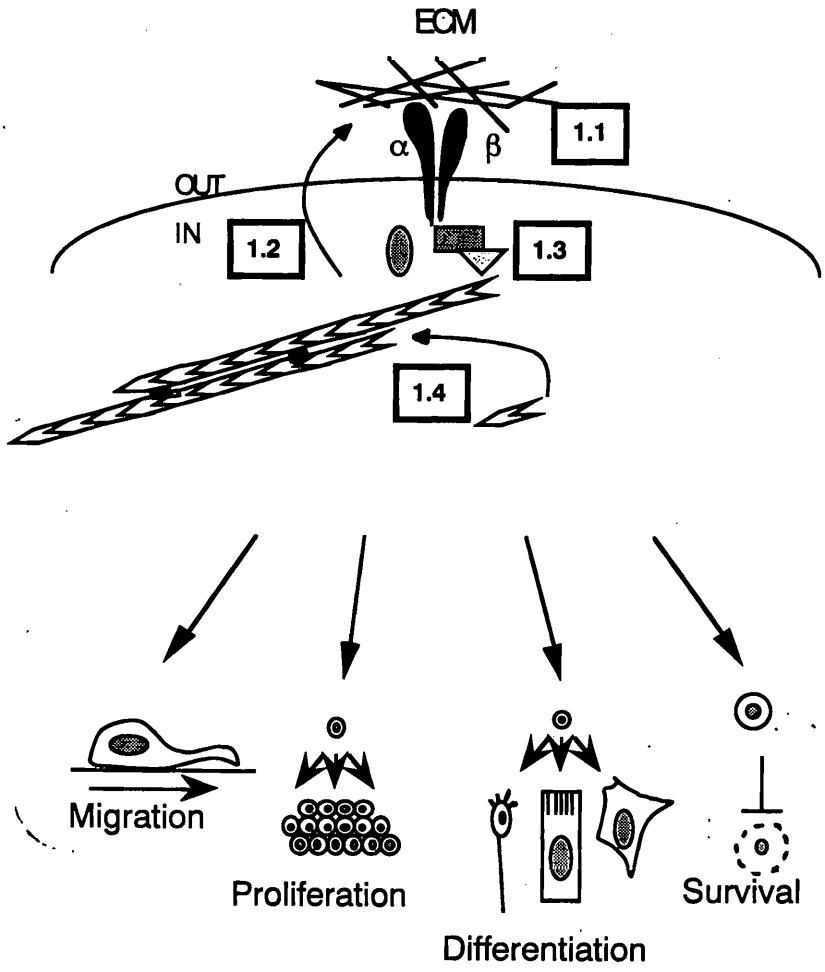


Figure 1.1. Integrin-based adhesion and its function in the biological processes.

Upon activation of integrins by the interaction with the ECM, focal adhesions are organised and connected to the actin cytoskeleton. Integrins are known to initiate signals for the reorganisation of the cytoskeleton, required for cell shape modification and for several important biological functions. Integrin affinity for the ligand can be regulated by an "inside-out" mechanism. The numbers in the boxes indicate the paragraphs were the various aspects of cell adhesion will be treated.

reorganisation of the actin cytoskeleton, to allow cells to change their morphology in different situations. For several cells in culture on ECM components, this interaction leads to the organisation of sites of tight adhesion, called focal adhesions. These structures are considered to be a valuable model to study the mutual interactions occurring between the ECM and the cytoskeleton. The molecular organisation of focal adhesions and their function will be analysed in 1.3. Focal adhesions are also known to contain signalling molecules for the reorganisation of the actin cytoskeleton. The molecular basis of actin cytoskeleton organisation and dynamics will be discussed in 1.4.

1.1 THE INTEGRIN RECEPTORS FOR THE ECM

1.1.1 Variety of integrins and ligands

The ECM is a composed of a variety of versatile glycoproteins and proteoglycans that are secreted locally and assembled into an organised meshwork in close association with the surface of the cell. It represents a dynamic and information-rich environment controlling several aspects of cell behaviour; in fact it has trophic and support functions, and it plays an active and complex role in regulating cell development, migration, proliferation, shape and function. The ECM has a complex molecular composition which vary both on the types of macromolecules and on the way they are organised. This gives rise to an amazing diversity of forms, each adapted to the functional requirements of the particular tissue. ECM molecules can be secreted by different types of cells, in particular by fibroblasts and other cells of the connective tissues. Some of the best known components of the ECM are collagens, laminins, fibronectin, vitronectin, thrombospondin and proteoglycans. Fibronectin is one of the most abundant ECM proteins secreted by chicken embryo fibroblasts (CEFs), the cell system utilised in this study. It is a large glycoprotein composed of two subunits joint by a pair of disulphide bonds, each of which contains 15 typical type III fibronectin repeats. Fibronectin assembles on the surface of cells as highly insoluble fibrils, composed by fibronectin dimers cross-linked to one-another by additional disulphide bonds.

The family of integrin receptors for the ECM is made by heterodimers composed of α and β transmembrane subunits, interacting by non covalent bonds. The association between the two subunits determines the specificity of the receptor for various molecules

INTEGRIN		LIGAND
β1*	α1	Laminin, Collagen
	α2	Laminin, Collagen, α3β1
	α3*	Laminin, Collagen, Fibronectin, Entactin, Vitronectin, α2β1, α3β1
	α4	Fibronectina, VCAM-1, MadCAM-1
	α5	Fibronectin
	α6*	Laminin
	α7*	Laminin
	α8	Fibronectin, Vitronectin, Tenascin
	αV	Fibronectin, Vitronectin
	α9	Osteopontin, Tenascin
β2	αL	ICAM-1, ICAM-2, ICAM-3
	αD	ICAM-3
	αM	iC3b, Fibrinogen, Factor X, ICAM-1
	αX	iC3b, Fibrinogen
β3*	αIIb	Fibrinogen, Fibronectin, vWFactor, Vitronectin, Thrombospondin
	αV	Fibrinogen, Fibronettina, vWFactor, Vitronettina, Thrombospondin
β4*	α6*	Laminin
β5	αV	Vitronectin
β6	αV	Fibronectin
β7	α4	Fibronectin, VCAM-1, MadCAM-1
	αE	E-cadherin
β8	αV	?

Table 1.1 The family of integrin receptors in vertebrates.

Asterisks indicate the subunits for which alternative splicing variants have been described. αE=α subunit specifically expressed in intraepithelial lymphocytes; iC3b=inactive component of the complement C3b; MadCAM-1=Mucosal Addressin Cell Adhesion Molecule.

of the extracellular matrix. Currently, the family of integrin receptors comprises 22 α/β heterodimers, obtained by several combinations of 8 different β and 16 different α subunits (Table 1.1). The existence for some α and β subunits of alternative splicing variants for both cytoplasmic and extracellular domains, further increases the complexity of the integrin family (see 1.1.2). As shown in Table 1.1, an integrin can often bind more than one ligand, and a single ligand can be recognised by more than one integrin. Most of the ligands in Table 1.1 are ECM molecules that mediate cell-substrate adhesion, but some, like fibrinogen, also mediate cell-cell interactions. Moreover, some integrins bind to integral transmembrane proteins of the immunoglobulin superfamily (ICAM, VCAM or MadCAM), mediating cell-cell interactions directly. The β1 subunit, by combining with 10 different α chains, constitutes the largest subfamily of integrin receptors. β1 integrins are the most important family of receptors for the ECM in CEFs (Bossy and Reichardt, 1990).

1.1.2 Integrin structure

Both α and β subunits of integrins are transmembrane glycoproteins composed by a large extracellular, N-terminal domain, a single transmembrane segment, and an usually short cytoplasmic, C-terminal domain (Fig. 1.2). Both the intracellular and the large extracellular domain (more than 75 KD for β and more than 100 KD for α subunits) are known to be involved in α and β interaction, which is believed to occur in a pre-Golgi compartment (Hynes *et al.*, 1989). The N-terminal portion of integrin α subunits comprises seven homologous, tandemly repeated domains of about 50 aminoacids (Fig.1.2 B). Repeats 4-7 (or in some integrins 5-7) contain cation-binding sequences, similar to the EF-hand motif found in Ca^{2+}-binding proteins, such as calmodulin. α subunits can be divided in two groups, on the basis of biochemical analysis and sequence comparison. The first group includes αV, α3, α6, and αIIb, and comprises α subunits processed by a post-translational proteolitic cut that separates them in a large extracellular portion covalently associated by a disulphide bond to a shorter portion (25-30 KD) including the transmembrane domain. Members of the second group of α subunits, including α1, α2, αE, αL, αX, αM, and αD, are not proteolitically processed and contain a domain (the A-, or I-domain) of around 200 aminoacids inserted between the second and the third N-terminal repeats. This domain is homologous in sequence to the A-domains of von Willebrand factor, and has been shown to contain a single cation

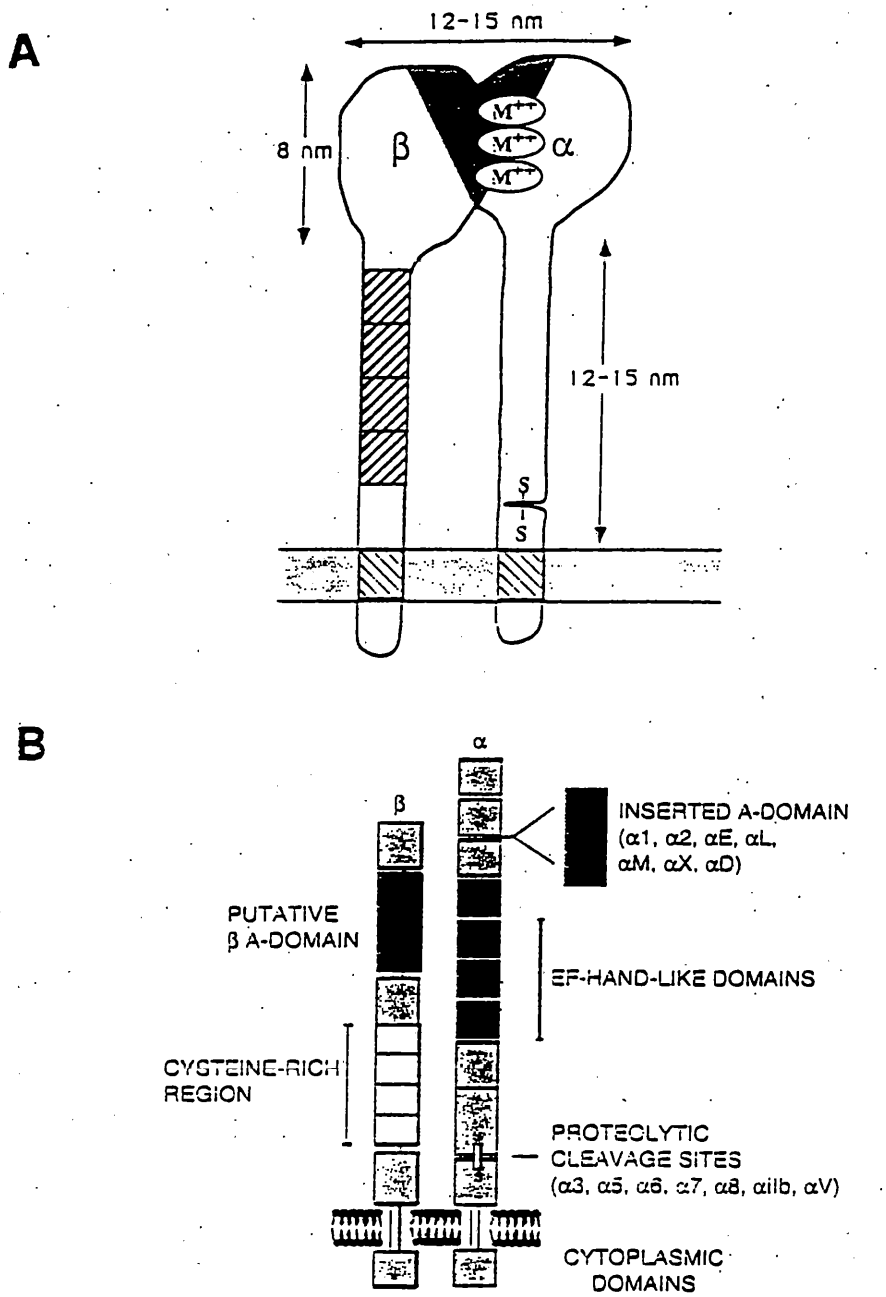


Figure 1.2. Structural features of integrin receptors.

Panel A: Overall shape of integrin receptors, as deduced from electron microscopy. The putative location of the cysteine-rich repeats of the β subunit (crosshatched), and the metal-binding sites in the α subunit (M^{++}), are indicated. The shaded area represents the ligand-binding region. (From Hynes, 1992).

Panel B: Schematic diagram of integrin structure (from Mould, 1996). See text for further details.

binding site (Lee *et al.*, 1995; Qu and Leahy, 1995). The extracellular portion of the β subunit contains some cystein-rich regions in the "stalk" region, with homology to EGF modules, probably involved in the formation of intramolecular disulphide bonds. Near the N-terminus, the β subunit contains a region of about 240 amino acids that is highly conserved between different β subunits. By comparison of consensus hydropathy plots or secondary structure prediction, this structure appears to contain an A-domain-like structure with a cation-binding site. This structure includes multiple conserved oxygenated residues critical for ligand binding, and non-conserved residues critical for ligand specificity (see below).

With the exception of β4 cytoplasmic domain, containing more than 1000 aminoacids, integrin cytoplasmic domains contain less than 60 aminoacids and they don't have any known enzymatic activity. The primary sequence of the β subunits is highly conserved during evolution, and in particular three clusters of residues are conserved among species (Fig. 1.3). The first cluster, which is unique to β1 integrins, is a stretch of 11 amino acids adiecient to the transmembrane domain that consists primarily of charged amino residues. The second cluster includes an NPXY sequence that has been associated with internalisation in the low density lipoprotein receptor (Chen *et al.*, 1990), and is conserved between all β subunits (with the exception of β4). The third cluster contains an NPXY-related sequence conserved between β1, β2 and β7, and is somewhat divergent in the β3, β5 and β6 subunits (Fig. 1.3). A number of isoforms for some of the β subunit cytoplasmic domains have been identified which arise from alternative splicing. In particular, 4 different alternative splicing variants have been described for the cytoplasmic tail of the β1 subunit: β1A, B, C, and D; only β1A (which is ubiquitous) and D (muscle specific) localise to focal adhesions. β1B is found in skin and liver, while β1C is found in non-proliferative cells and it has been suggested to have an inhibitory effect on cell cycle progression (see Fornaro and Languino, 1997, for a review).

Like the β subunit cytoplasmic domains, the α subunit cytoplasmic domains are highly conserved among different species. However, in contrast to the β subunits, the different α subunit cytoplasmic domains show little homology among one another, suggesting distinct roles. The most striking conserved motif is the sequence GFFKR, which is adjacent to the transmembrane domain and is conserved among all α subunits. At the C-terminus, the sequence TSDA is conserved among the α5, α6, αV and α8 subunits and is partially conserved in the α3 subunit, but its exact function is not clear yet. Like the β subunits, some α subunit cytoplasmic domains present variants obtained

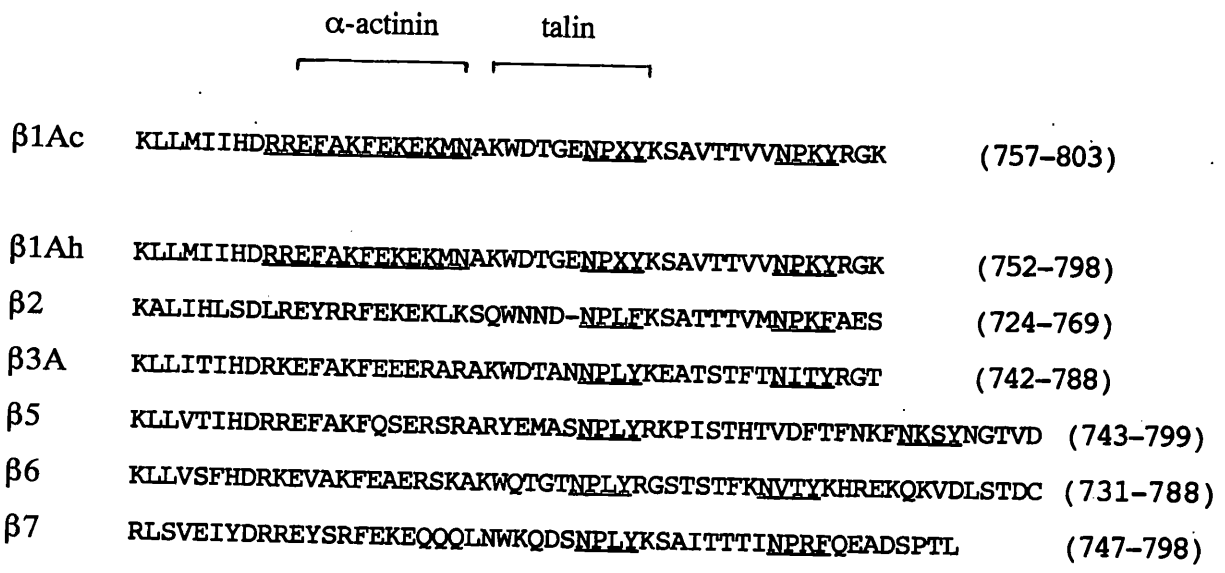


Figure 1.3 Aligment of the cytoplasmic domains of β integrins.

Sequence alignment of the integrin β subunit cytoplasmic domains from the membrane proximal lysine. β1A is one of four cytoplasmic domain splice variants; β1Ac represents the chicken cytoplasmic domain sequence, and β1Ah is the equivalent human sequence. All other sequences are from human. The residues numbers are in parentheses. The three major conserved clusters are underlined. The major binding site for α-actinin (FAKFEKEKMN) and talin (WDTGENPIYK) in the chicken β1 integrin cytoplasmic domain, as determined using *in vitro* binding assays, are indicated.

from alternative splicing. The splicing variants are often expressed in a tissue-specific manner, but their exact biological function is not completely clear.

1.1.3 The structural basis of integrin-ligand interactions

Integrin ligand-binding sites within integrin αβ heterodimers have been identified by a combination of approaches, including chemical cross-linking of ligand peptides, synthesis of recombinant integrin fragments and peptides, functional mAb epitope-mapping, and analysis of natural and engineered integrin mutants (Humphries *et al*, 1994; Loftus *et al*, 1994). These studies have implicated three regions in ligand binding: first, sequences close to, or at EF-hand-like motifs in the N-terminal part of the α subunit; second, the A-domain that is found in a subset of α subunits; and third, the A-domain-like structure present near the N-terminus of the β subunit (see previous paragraph). The ligand-binding pocket appears therefore to involve both subunits and the interaction is likely to be complex in nature. The seven sequence repeats present in the N-terminal region of the α subunits have been proposed to arrange in a β-propeller model, in which seven four-stranded β-sheets are organised in a torus around a pseudo-symmetry axis (Springer, 1997). Several predicted loops critical for ligand binding have been identified in the upper face of the proposed β-propeller model, in the face opposite to the cation-binding site (Fig. 1.4). If an A-domain was present in the α subunit, it would be inserted between the second and the third of the propeller blades. The structure of the A-domain from several α subunits have now been solved (Lee *et al.*, 1995; Qu and Leahy, 1995; Qu and Leahy, 1996). It adopts a Rossmann, or dinucleotide-binding fold, with a central sheet of β-strands decorated peripherally by six α-helices. At one end of the module, a series of loops coordinate a divalent cation. The A-domain-like structure present in the N-terminal domain of the β subunits, has also been implicated in ligand recognition (Fig. 1.4). Although the overall structure of integrin domains can not be predicted, their arrangement relative to each other and to the ligand, together with a transition from inactivity to activity, has been hypothesized for α5β1 integrin binding to fibronectin (Humphries and Newham, 1998), and it is described in Fig. 1.4.

All integrin-ligand interactions are divalent cation dependent and, consistent with this finding, all three of the regions above that are implicated in ligand binding have been shown to contain sequences that interact with divalent cations. As large integrin ligands frequently employ a short acidic peptide as a key binding determinant (e.g. RGD, present in several ECM molecules), it has been suggested that a receptor-bound cation might

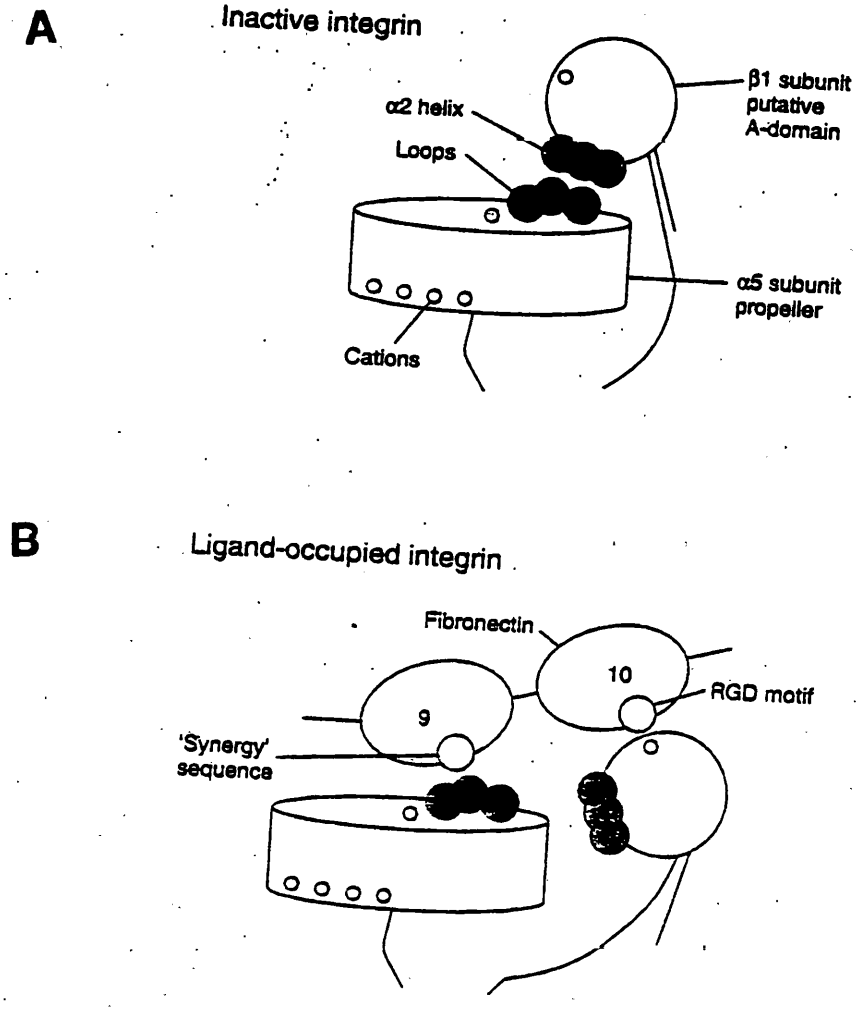


Figure 1.4. A speculative model for the structure of α5β1 integrin ligand-binding site, based on homology to heterotrimeric G-proteins.

Panel A: in the inactive form of the integrin, the α2 helix of the β1 subunit putative A-domain interacts with loops projecting from the top surface of the α5 subunit β-propeller. Panel B: conformational changes in the α2 helix, induced by cation-binding or addition of stimulatory mAbs that recognise this region, elicit a major repositioning of the integrin subunits, an opening of the integrin dimer and a concomitant exposure of ligand-binding sites in the α5 subunit. The RGD motif in type III repeat 10 of fibronectin then engages the bound cation in the βA-domain, and the synergy sequence (PHSRN) present in type III repeat 9 engages a site in the α5 subunit β-propeller (from Humphries and Newham, 1998).

function as an integrin-ligand bridge (Humphries *et al.*, 1994). The cation dependence of ligand binding has been investigated for a number of integrins, and a similar, though not exclusive, specificity has been observed: Mn^{2+} and Mg^{2+} usually promote ligand binding, while Ca^{2+} has usually an inhibitory effect (Tuckwell *et al.*, 1992). Integrins are believed to exist in an equilibrium between at least two different conformations: one with a low affinity for the ligand (inactive) and one with a high affinity for the ligand (active). Some cations, like Ca^{2+}, have been suggested to displace the conformational equilibrium in favour of a conformation that is unable to bind ligand, whereas others, like Mg^{2+} or Mn^{2+}, displace the equilibrium in the opposite direction and increase the proportion of integrin that is competent to bind ligand (Mould, 1996). A large number of anti-integrin mAbs have been reported which either perturb or enhance ligand binding. In this scenario, stimulatory mAbs may act by increasing the proportion of the integrin population that is in a ligand-competent state, either by displacing the conformational equilibrium or by directly inducing changes in integrin shape. On the contrary, inhibitory mAbs stabilise an integrin conformation that has a low affinity for the ligand, or directly induce the formation of such a conformation (Humphries, 1996).

1.2 INSIDE-OUT REGULATION OF INTEGRIN AFFINITY

1.2.1 The biological importance of integrin affinity regulation

Cells have the ability to rapidly modulate binding affinity of the integrins for their ligands, a process known as "affinity regulation". This process allows the cell to modulate the strength of adhesion and adds extraordinary flexibility to integrins. Ligand-binding regulation can be achieved by cells by using both integrin affinity modulation and integrin clustering (avidity modulation). Integrin clustering can be regulated by intracellular signalling events that remodel cytoskeletal linkages, such as the ones occurring at focal adhesion sites (see 1.3), or alter receptor diffusion rates within the cell membrane. Changes in both integrin affinity and avidity are not mutually exclusive, but the relative contributions of each to ligand binding vary with integrin type and the cellular context (Hato *et al.*, 1998; Stewart and Hogg, 1996; Lub *et al.*, 1995). Thus far, integrins of the β1, β2, β3 and β7 families have been shown to modulate their ligand-binding affinities in response to cytoplasmic signals (Faull *et al.*, 1993; Crowe *et al.*, 1994; Shattil *et al.*, 1998). Platelet aggregation is a classical example of a physiological

process dependent on integrin affinity modulation. The aggregation of platelets relies on their crosslinking by fibrinogen, a soluble plasma protein that binds to the integrin αIIbβ3. Resting platelets are unable to aggregate because their αIIbβ3 is in a partially active conformation that has low affinity for soluble fibrinogen. Following platelet activation by specific agonists, αIIbβ3 undergoes a conformational change that dramatically increases its binding to fibrinogen, resulting in platelets aggregation (Shattil *et al.*, 1998).

Another example of integrin affinity modulation concerns cell migration, which is dependent on rapid, controlled changes in integrin-dependent cell adhesion, suggesting that coordinated activation and de-activation of integrins play a crucial role in this process (Lauffenburger and Horwitz, 1996). Indeed, the importance of integrin affinity modulation as a crucial regulator in cell migration is illustrated by the observation that locking integrins in a constitutive high affinity state can inhibit cell migration (Kuijpers *et al,* 1993; Huttenlocher *et al.*, 1996). Significantly, a role for integrin affinity modulation in embryonic development is starting to emerge. Martin-Bermudo *et al.* (1998) have recently demonstrated in the Drosophila embryo that normal morphological development is disrupted by a constitutively active variant of the αPS2 subunit.

1.2.2 Integrin cytoplasmic domains play a central role in affinity modulation

Integrin affinity modulation is proposed to involve the propagation of conformational changes from the integrin cytoplasmic domain to the extracellular ligand-binding sites, leading to a direct increase in ligand-binding affinity.

The role of integrin cytoplasmic domains in activation has been demonstrated by expression and analysis of recombinant integrins. The membrane proximal regions of β and α subunit cytoplasmic domains share a similar organisation of polar and apolar amino acids just after the membrane-cytoplasm interface. The conserved sequences for the α and β subunits are GFFKR and LLv-iHDR, respectively, and the deletion of these sequences in either the α or β subunit activates integrins (Crowe *et al.*, 1994; O'Toole *et al,* 1994). These sequences form a structural constraint, referred to as the membrane-proximal "hinge", that locks the integrins in the low affinity conformation. Sequences C-terminal to the membrane proximal motifs in the α and β subunit cytoplasmic domains also play a crucial role in integrin activation. In fact, the deletion of sequences C-terminal to the GFFRK motif in a number of distinct α cytoplasmic domains inhibit cell adhesion,

suggesting that these sequences are required for integrin activation (Williams *et al.*, 1994). The well-conserved NPXY motif in the β cytoplasmic domain appears to be essential for integrin activation (O'Toole *et al.*, 1995). Together these observations lead to the idea that the interactions of α and β cytoplasmic domains with specific regulatory factors could modulate integrin affinity. This hypothesis is supported by the observation that the transfection of either isolated β1 or β3 cytoplasmic domains induces a dose-dependent decrease in the ligand-binding affinity of active αIIbβ3 chimeras (Chen *et al.*, 1994), a process referred to as "dominant-suppression". This suggests that isolated cytoplasmic domains compete for limiting amounts of intracellular factors required for integrin activation.

The presence of highly conserved tyrosine, threonine and serine residues in integrin cytoplasmic domains suggests that phosphorylation has the potential to regulate integrin activation. Indeed, there is some evidence that suggests that the phosphorylation of tyrosine residues in the β3 cytoplasmic domain plays a role in the activation of αVβ3 (Blystone, *et al.*, 1996). However, detailed studies on αIIbβ3, αLβ2 and α6Aβ1 have thus far failed to demonstrate a potential role for phosphorylation in the activation of these integrins (Williams *et al.*, 1994).

1.2.3 Intracellular signalling pathways involved in affinity modulation

At present, the cytoplasmic signalling pathways regulating integrin affinity are poorly understood. During platelet aggregation, agonists such as thrombin, activate αIIbβ3 via heterotrimeric G-protein-coupled receptors, which in turn, stimulate phospholipase C, resulting in phosphoinositide hydrolysis and activation of protein kinase C (Shattil *et al.*, 1998). Experiments with inhibitors of various protein tyrosine kinases or phosphatases suggest that changes in protein phosphorylation play a key role in the activation of integrins in platelets (Shattil *et al.*, 1992). A number of recent studies in cells other than platelets indicate that the Ras family of small GTP-binding proteins and their effectors play a crucial role in regulating integrin affinity. Activated H-Ras and its kinase effector Raf-1 inhibit integrin activation in Chinese hamster ovary (CHO) cells (Huges *et al.*, 1997). R-Ras, a small GTP-binding protein highly homologous to H-Ras, is also a regulator of integrin activation but, unlike H-Ras, activated R-Ras stimulates rather than inhibiting integrin ligand-binding affinity (Zhang *et al.*, 1996). This raises the possibility that these small GTP-binding proteins act in concert to modulate integrin

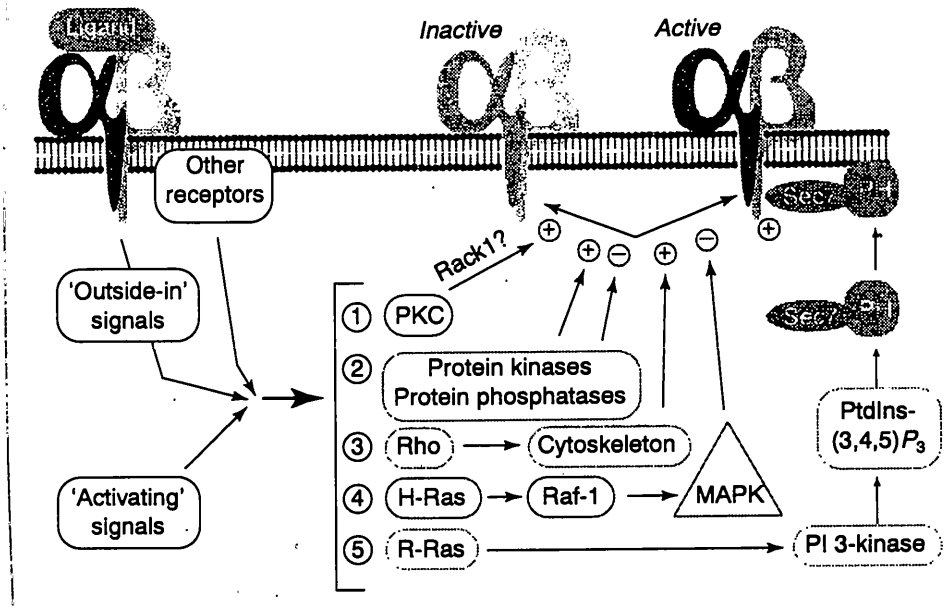


Figure 1.5. Signalling pathways implicated in integrin affinity modulation.

In most cases, it is unclear how the depicted pathways are directly linked to a molecular interaction with the integrin. Protein Kinase C (PKC) might act via its substrate Rack 1, which binds directly to integrin β tail. Phosphoinositide (PI) 3-kinase might activate integrins through members of a novel protein family consisting of a Sec7- and a pleckstrin-homology (PH) domain, a member of which is cytohesin-1. After phosphatidylinositol (3,4,5)-triphosphate [PtdIns(3,4,5)P_3]-induced translocation to the membrane, these proteins might interact directly with integrin β cytoplasmic domains to modulate ligand-binding affinity. Symbols and abbreviations: +, a positive regulator of activation; -, a negative regulator of activation; PKC, Rack1, receptor for activated protein kinase C; MAPK, mitogen activated protein kinase; Sec7, Sec7-homology domain. From Huges and Pfaff, 1998.

affinity (Fig. 1.5). There is also evidence suggesting that the small GTP-binding protein Rho plays a role in integrins activation. Inactivation of Rho by C3 exoenzyme blocks α4β1-dependent adhesion of chemoattractant-stimulated lymphocytes to VCAM-1 and αLβ2-dependent adhesion of neutrophils to fibrinogen (Laudanna *et al.*, 1996). Recent studies on integrin function in leukocytes have identified a potentially important role for PI 3-kinase in regulating the activation of β1 and β2 integrins (Shimizu and Hunt, 1996). Interestingly, R-Ras can stimulate PI 3-kinase activity, providing a plausible explanation for an involvement of R-Ras in integrin activation (Marte and Downward, 1997).

A potential link between PI 3-kinase and the activation of β2 integrins is emerging from the finding that the PI 3-kinase product phosphoinositide (3,4,5)-trisphosphate binds to certain pleckstrin-homology (PH) domains, leading to the recruitment of the PH domain to the plasma membrane (Lemmon *et al.*, 1997). Cytohesin-1, which interacts with the cytoplasmic domains and subsequently activates αLβ2, contains a PH domain (Kolanus *et al.*, 1996). Therefore it is possible to envisage a scenario in which PI 3-kinase activation results in the membrane localisation of cytohesin-1 (Fig. 1.5). Subsequently, cytohesin-1 could then bind to the β2 subunit cytoplasmic domain and induce activation. It remains to be determined whether the cytoplasmic domains of other β subunits can bind to other cytohesin-like proteins.

1.2.4 Direct interactions of proteins with integrin cytoplasmic domains

Given the importance of integrin cytoplasmic tails in integrin activation, proteins that interact directly with integrin cytoplasmic domains are potentially excellent candidates for regulators of activation. Consequently, there has been an intense effort in the last few years to identify such proteins. This has resulted in a growing list of proteins that can bind to integrin cytoplasmic domains, at least *in vitro*, including both cytoskeletal and potential regulatory proteins (Table.1.2). However, it should be stressed that solid evidence for an occurrence of these interactions *in vivo* and for their implication in affinity modulation is mostly lacking. Interactions of integrins with the cytoskeleton via talin (Horwitz *et al.*, 1986) and α-actinin (Otey *et al.*, 1990) are thought to occur as a consequence of integrin-ligand engagement, contributing to the formation of focal adhesion complexes (see 1.3). It is unclear whether such interactions also occur independently of ligand binding to regulate receptor affinity. However, recent evidence

suggests that interactions of integrin cytoplasmic domains with the cytoskeleton can lock integrins in their inactive state (Stewart *et al.*, 1998).

Calreticulin has been shown to bind to the highly conserved and functionally important GFFKR motif in integrin α tails (Rojiani *et al.*, 1991). This interaction is increased upon integrin stimulation and proposed to be required for the stabilisation of the high affinity state of the receptor (Coppolino *et al.*, 1995). Moreover, a potential role for calreticulin in affinity modulation is illustrated by the defects of calreticulin-null embryonic stem cells in integrin-mediated cell adhesion and Ca^{2+} influx (Coppolino *et al.*, 1997). Focal adhesion kinase (p125 FAK) and paxillin also interact directly with integrin β cytoplasmic domains peptides *in vitro* (Schaller *et al.*, 1995). However, a role for these interactions in regulating integrin affinity *in vivo* has yet to be determined. A serine/threonine protein kinase (p59 ILK), which binds to β1, β2 and β3 cytoplasmic tails, disrupts cell-substratum adhesion when overexpressed in cells, indicating a possible role in integrin affinity modulation (Hannigan *et al.*, 1996). The receptor for activated protein kinase C (Rack 1), a Trp-Asp (WD) repeat protein, binds to the conserved membrane-proximal region of β1, β2 and β5 subunit cytoplasmic domains (Liliental and Chang, 1998). The association of Rack 1 in vivo appears to depend upon prior stimulation with phorbol esters, suggesting that Rack 1 could potentially act as link between protein kinase C (PKC) and integrins. Recently, several less ubiquitous proteins have been identified that interact with specific integrin subunit cytoplasmic domains: β3-endonexin, which is specific for β3 integrin tails (Shattil *et al.*, 1995), cytohesin-1, a protein binding to the cytoplasmic domain of β2 integrins (Kolanus *et al.*, 1996), a Ca^{2+}-and integrin-binding protein (CIB) interacting with the integrin αIIb tail in platelets (Naik *et al.*, 1997), and ICAP-1, a novel 200 amino acid protein that interacts specifically with the β1 integrin cytoplasmic domain (Chang *et al.*, 1997). The role of CIB and ICAP-1 in integrin activation is unknown, but there is evidence for a role for β3-endonexin and cytohesin-1 in the regulation of integrin affinity (Kolanus *et al.*, 1996; Kashiwagi *et al.*, 1997).

Finally, lateral associations of integrins with membrane proteins might also modulate integrin affinity, not necessarily through interactions with integrin cytoplasmic domains. The transmembrane protein CD98, and early T-cell activation antigen that associates with "active" β1 integrins, was implicated as a regulator of integrin activation (Pfaff *et al.*, 1998). β3 integrins can associate with CD47 or IAP (integrin associated protein), and it has been shown recently that binding of IAP to its ligand thrombospondin can activate platelet integrin αIIbβ3 (Chung *et al*, 1997). Members of the tetraspanning

Binding protein	Tail partner	Detection	Features
Filamin	β_{1A}, β_2, β_7	COIP, PEP	Structural cytoskeletal protein
Talin	β, α_{IIb}	PEP, EQ	Structural cytoskeletal protein
α-Actinin	β	PEP, INT	Structural cytoskeletal protein
CIB	α_{IIb}	2HYB	Has homology to calcineurin B; contains two EF-hand motifs
Calreticulin	α	PEP, COIP	Expression correlates with integrin-mediated cell adhesion
pp125^{FAK}	β	PEP	A tyrosine kinase localized to focal adhesions
pp59^{ILK}	β	2HYB	Contains a serine-threonine kinase domain; overexpression inhibits cell adhesion
Paxillin	β	PEP	An adaptor protein containing SH2- and SH3-binding motifs and LIM domains
Cytohesin-1	β_2	2HYB	Contains Sec7 and PH domains. Overexpression increases $\alpha_L\beta_2$-mediated adhesion
β_3-Endonexin	β_3	2HYB	Overexpression activates $\alpha_{IIb}\beta_3$
ICAP-1	β_1	2HYB	Ligation of β_1 integrins induces phosphorylation of ICAP-1
Rack1	β	2HYB	Interaction with integrins *in vivo* is PMA dependent

Table 1.2. Proteins interacting directly with integrin cytoplasmic domains.

Abbreviations: CIB, calcium- and integrin-binding protein; COIP, co-immunoprecipitation; EQ, equilibrium gel filtration; FAK, focal adhesion kinase; ICAP, integrin cytoplasmic domain-associated protein; ILK, integrin-linked kinase; INT, binding to purified integrin; PEP, synthetic/recombinant peptide studies; PH, plekstrin-homology domain; Sec7, Sec7-homology domain; SH2, Src-homology 2 domain; SH3, Src-homology 3 domain; 2HYB, yeast two-hybrid. (Modified from Huges and Pfaff, 1998).

class of transmembrane (TM4) proteins, including CD9, CD81, NAG-2 and CD63, have been shown to associate with certain integrins, but to date there is no evidence that these interactions contribute to the regulation of integrin affinity (Hemler *et al.*, 1996; Indig *et al.*, 1997).

Currently, it is not clear how "activating" signals acting on integrin cytoplasmic tails can mediate changes in integrin ligand-binding affinity. Current experimental data suggest that integrins are present on the surface of the cell surface in an default inactive state, with the membrane-proximal hinge providing a constraint that locks the receptor in a resting conformation. Activation could then be initiated by the interaction of cytoplasmic factors with the integrin cytoplasmic tails, thus inducing changes in the spatial relationship of the α and β tails, breaking the membrane proximal hinge. This initial conformational change would then cross the lipidic bilayer to the extracellular domain of integrin, ultimately changing its affinity for the ligand (see Fig. 1.4).

1.3 INTEGRIN SIGNALLING: THE FOCAL ADHESION COMPLEX

1.3.1 Signalling from cell-substrate adhesion complexes

It has been known for many years that, upon interaction with their extracellular ligands, integrin receptors are able to transduce signals inside the cell. Protein phosphorylation is one of the earliest events detected in response to integrin stimulation (Schaller and Parson, 1993), and it will be further discussed in 1.3.4. Integrin engagement can induce an increase in the intracellular [Ca^{2+}], a key regulator of intracellular signalling via several integrins (Ingber *et al.*, 1990; Hendey and Maxfield, 1993;, Shankar *et al.*, 1993; Zimolo *et al.*, 1994; Sjaadtad *et al.*, 1996; Lawson and Maxfield, 1995). Numerous phospholipid metabolites generated in response to integrin engagement also function in integrin-mediated signal transduction, in part by activating PKC (Divecha and Irvine, 1995), in part by regulating the function of cytoskeletal proteins such as profilin, gelsolin, α-actinin, or vinculin (Janmey, 1994). Integrins engagement causes elevation of the intracellular pH, as a result of the activation of a proton antiporter, and this accompanies cell spreading and appears to be regulated by several different integrins (Schwartz and Ingber, 1994).

Since integrins possess short cytoplasmic tails, and like the T-cell receptor they have no intrinsic enzymatic activity, their signalling strategies must be distinct from those of growth factor and other receptors with documented kinase or phosphatese activities in their cytoplasmic domains. Several evidences support the idea that the mechanism of signalling mediated by integrins depends critically on their ability to assemble a cytoskeletal framework for the effective interaction of components of the intracellular signalling machinery. Therefore, focal adhesions provide a valuable model system for studying both integrin-mediated signal transduction and cytoskeletal transmembrane linkages. They are present on fibroblasts, and some other cells, which are well spread on ECM substrates, and they are a characteristic of β1 and β3 integrins. They are found primarily at cell borders where bundles of actin filaments terminate, and contain a very large number of different proteins. Whereas focal adhesions are seldom seen *in vivo*, and are only readily apparent under certain conditions, it is assumed that comparable molecular structures exist in tissue. In addition to major structural components such as α-actinin, talin, and vinculin (discussed in 1.3.3), focal adhesions in spread fibroblasts also contain several additional non-enzymatic proteins that are present at low levels, suggesting that they may play a regulatory role (Fig. 1.6). These include paxillin (see also 1.3.3), which is a major substrate for tyrosine kinases, and tensin, which has a src homology 2 (SH2) domain (Davis *et al.*, 1991; Lo *et al.*, 1994). As SH2 domains recognise phosphorylated tyrosine residues on proteins, they could potentially link regulatory and structural proteins at focal adhesion sites. Finally, focal adhesions contain enzymatic regulatory molecules that become associated with the cytoskeleton as it is reorganised during cell spreading. These include the protein tyrosine kinase $pp125^{FAK}$ (FAK, focal adhesion kinase) (Schaller *et al.*, 1992), the protein kinase C (Barry and Critchley, 1994), $pp60^{src}$ (Clark *et al.*, 1994; Schlaepfer *et al.*, 1994), a Ca^{2+}-dependent proteolitic enzyme (calpain II) (Beckerle *et al.*, 1987), and perhaps components of the Rho family of small GTP-binding proteins.

1.3.2 The role of integrin cytoplasmic domains in the association with the cytoskeleton

Several lines of evidence point to the integrin β cytoplasmic domain as the structure that targets integrins to focal adhesions. Deletion of the β1 cytoplasmic domain prevents the localisation of the truncated chicken β1 integrin to focal adhesions when transfected in NIH 3T3 fibroblasts (Hayashi *et al.*, 1990). The use of chimeric receptors

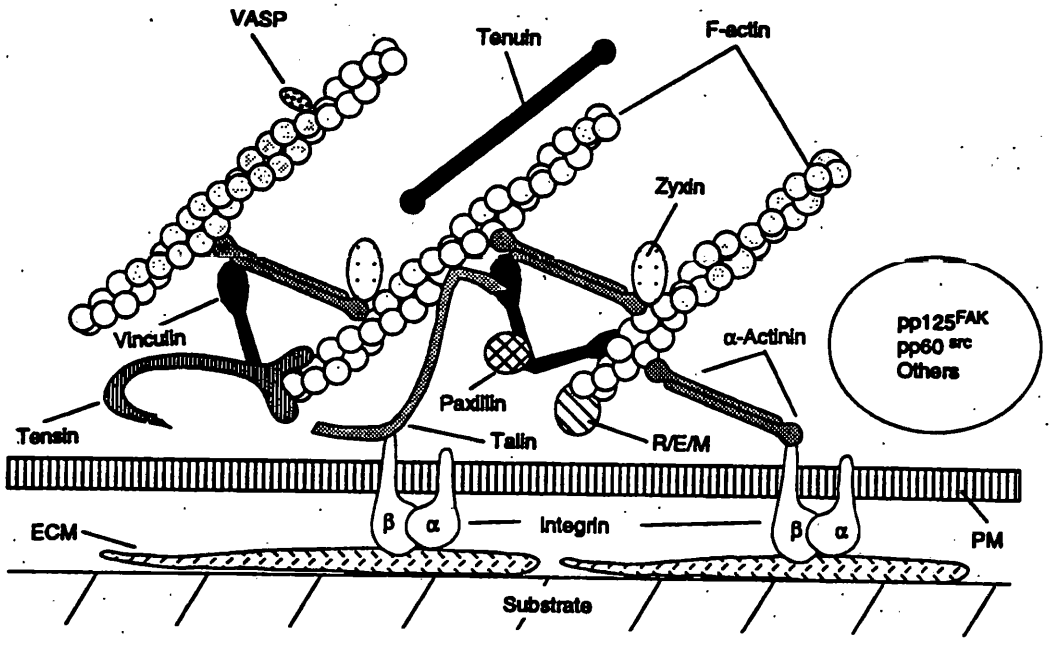


Figure 1.6 Model of the major protein-protein interactions in focal adhesions.

The model has been obtained from colocalisation studies and from reconstitution of some interactions *in vitro*, altough most associations have not been verified *in vivo* yet. Abbreviations are: ECM, extracellular matrix; PM, plasma membrane; R/E/M, member of the radixin/ezrin/moesin family; VASP, vasodilatator-stimulated phosphoprotein. (From Luna and Hitt, 1992).

containing the cytoplasmic domain of the β1 or β3 integrin subunit fused to the transmembrane and extracellular domains of the interleukin-2 receptor has demonstrated directly that targeting information is contained in the β cytoplasmic domain (LaFlamme *et al.*, 1992; LaFlamme *et al.*, 1994). When overexpressed, these chimeric proteins behave as dominant-negative mutants, blocking both the adhesive functions of integrins and the signalling induced by ligand binding (LaFlamme *et al.*, 1994; Smilenov, *et al.*, 1994). Several complementary studies point to all three conserved clusters in the β1 cytoplasmic tail (see 1.1.2 and Fig. 1.3) for localisation to focal adhesions. They include site-directed mutagenesis, cytoplasmic domain swaps, homologies among β subunits that localise to focal adhesions, and *in vitro* binding studies with synthetic peptides corresponding to cytoplasmic domain sequences (Marcantonio *et al.*, 1990; Takada *et al.*, 1992; Vignoud *et al.*, 1997; Reszka *et al.*, 1992; Filardo *et al.*, 1995; O'Toole *et al.*, 1995). β integrin cytoplasmic domains are known to bind to the different cytoskeletal proteins, including α-actinin and talin (Horwitz *et al.*, 1986; Pfaff *et al.*, 1998; Otey *et al.*, 1990; Schaller *et al.*, 1995), and different lines of evidence support the idea that activated integrins do associate with the actin cytoskeleton (Fox *et al.*, 1993; Felsenfeld *et al.*, 1996; Choquet *et al.*, 1997). The use of beads coated with integrin ligands and anti-integrin antibodies has revealed a hierarchy of accumulation of molecules at adhesive sites (Miyamoto *et al*, 1995a; Miyamoto *et al.*, 1995b). For example, pp125^{FAK} and tensin accumulate on the cytoplasmic side of the plasma membrane after simple clustering of β1 integrins, accompanied by a number of signalling molecules, including members of the Src family, Rho family GTPases, members of the lipid signalling and mitogen-activated protein kinase pathways, in a process that also required protein tyrosine phosphorylation. On the other hand, a number of cytoskeletal molecules, including α-actinin, talin and vinculin, require both integrin clustering and receptor occupancy by a ligand to accumulate in adhesion complexes, suggesting that, to recruit cytoskeletal proteins, the integrin must be in the ligand-bound state.

There is current evidence that integrin α-subunits play a negative role in regulating integrin association with the actin cytoskeleton. In fact, the cytoplasmic domain of the α-subunit is thought to mask the β integrin cytoplasmic domain and to prevent its localisation to focal adhesions by default. When the heterodimer is in the ligand-bound state, the two cytoplasmic domains move apart to reveal binding sites for cytoplasmic proteins (Ylanne *et al.*, 1993; O'Toole *et al.*, 1994; Huges *et al.*, 1996).

1.3.3 The major structural components of focal adhesions

The best characterised structural proteins mediating the connection between integrin receptors and the actin cytoskeleton are talin, α-actinin and vinculin (see Fig. 1.6). Talin is the first cytoplasmic protein found to interact *in vitro* with β1 integrins (Horwitz *et al.*, 1986), and the interaction requires both NPXY motifs of the chicken β1 subunit (Reszka et al, 1992). Talin appears to bind as a homodimer, providing a potential mechanism by which it might cross-link integrins (Vignoud *et al.*, 1997). In addition to binding to integrins, talin has three binding sites for vinculin (Gilmore *et al.*, 1993), and at least three binding sites for actin (Bolton *et al.*, 1997) (Fig.1.7A). Interestingly, talin has also been reported to possess actin nucleating activity (Kaufmann *et al.*, 1991). The fact that talin is recruited to newly forming focal adhesions before vinculin (DePasquale and Izzard, 1991), suggests that it might play a role in both the recruitment of pre-existing actin filaments to the cytoplasmic domain of integrins and *de novo* assembly of actin filaments (see also 1.4). Talin is also known to interact with charged lipids and to membranes (Niggli *et al.*, 1994). The N-terminal domain of talin contains regions of identity with the ezrin, radixin, moesin (ERM) family of proteins (Vaheri *et al.*, 1997), which are known to contain cryptic F-actin binding sites, hidden in an intramolecular association. These sites are regulated by phosphatidylinositol 4,5-bisphosphate (PIP_2), as well as by Rho family GTP-binding proteins (Hirao *et al.*, 1996; Mackay *et al.*, 1997). It is still to be estabilished whether a similar mechanism of regulation occurs for talin.

The actin cross-linking protein α-actinin provides another potential link between integrins and the actin cytoskeleton. α-actinin is a rod-shaped, anti parallel homodimer, with an N-terminal globular head containing the actin-binding site, a central region with four spectrin-like repeats which are responsible for the formation of the α-actinin homodimer, and a C-terminal region containing two EF-hand-like calcium-binding motifs (Flood *et al.*, 1995; Critchley *et al.*, 1999)(Fig. 1.7B). There are muscle and non-muscle isoforms of α-actinin, and only the non-muscle ones are able to bind calcium which inhibits their F-actin crosslinking activity (Imamura *et al.*, 1994). *In vitro*, α-actinin binds to peptides derived from integrin β1 cytoplasmic domains via undefined sequences within the spectrin-like repeats (Otey *et al.*, 1990; Otey *et al.*, 1993) and to vinculin via the fourth repeat (McGregor *et al.*, 1994). Zyxin, another focal adhesion component, binds to the N-terminal globular head of α-actinin (Crawford *et al.*, 1992). Also components of signalling pathways bind α-actinin, including the p85a subunit of

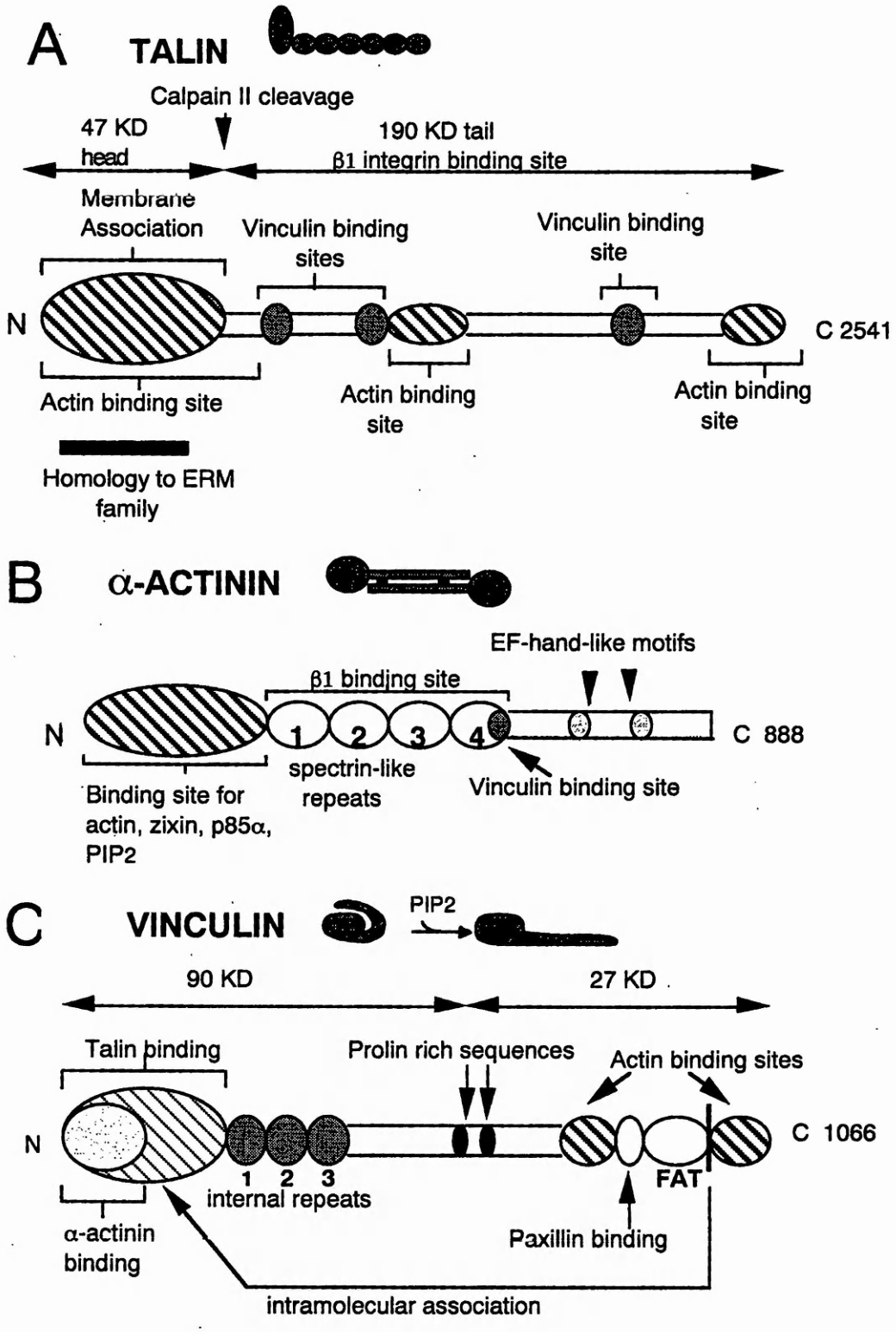


Figure 1.7 Structural features of the cytoskeletal proteins talin (A), α-actinin (B) and vinculin (C).

Abbreviations: FAT, focal adhesion targeting sequence; p85a, p85 subunit of phosphoinositide 3-Kinase. N and C indicate N- and C-termini respectively, with the residues number indicated. Double-headed arrows indicate proteolitic fragments of the protein.

phosphoinositide 3-kinase (Shibasaki *et al.*, 1994) and protein kinase N (Mukai *et al.*, 1997). In addition, α-actinin can bind the acidic phospholipid PIP2 (Fukami *et al.*, 1992) which can regulate its F-actin binding activity. The importance of α-actinin for focal adhesion and stress fibers integrity is suggested by microinjection experiments of proteolitic fragments of α-actinin into fibroblast, resulting in disruption of focal adhesions and actin stress fibers (Pavalko and Burridge, 1991), and by down-regulation experiments of α-actinin levels by antisense mRNA, which resulted in cells with fewer, smaller, focal adhesions and stress fibers (Gluck and Ben-Zeiev, 1994).

Vinculin is a well characterised cytoskeletal protein composed of an N-terminal globular head and a C-terminal rod-like domain or tail (Fig. 1.7C). *In vitro* studies have identified numerous binding sites within vinculin for other focal adhesion proteins, including talin and α-actinin, which bind to regions in the N-terminal head, and paxillin and actin, which bind to the C-terminal tail. Vinculin can bind also the vasodilatator-stimulating protein (VASP), tensin, and protein kinase C (PKC) (rewieved by Critchley *et al.*, 1999). Vinculin shows an intramolecular interaction between residues within the C-terminal region and the N-terminal head, which inhibits the binding of vinculin to several of its ligands (Johnson and Craig, 1994). Interestingly, this interaction is inhibited *in vitro* by acidic phospholipids, such as PIP2 (Gilmore and Burridge, 1996). Both microinjection experiments with anti-vinculin mAbs (Westmeyer *et al.*, 1990), and reduction of the level of expression of vinculin (Rodriguez Fernandez *et al.*, 1993), caused a reduction in number of focal adhesions and stress fibers, suggesting that vinculin stabilises focal adhesions and their link to actin stress fibers.

1.3.4 The role of phosphorylation in focal adhesion assembly

For many cells in culture, focal adhesions are the sites with the highest concentration of phosphotyrosine. The relationship between tyrosine phosphorylation and focal adhesion assembly is complex. Tyrosine phosphorylation is known to be an early event in integrin-mediated signalling (Schaller and Parson, 1993), and evidence exists that this phosphorylation has a role in focal adhesion assembly (Burridge *et al.*, 1992). Several tyrosine kinases have been identified in focal adhesions, including members of the Src family, C-terminal Src kinase (CSK), and the focal adhesion kinase (FAK). The latter appears to be the most abundant and becomes tyrosine-phosphorylated and activated in response to integrin-mediated adhesion (Kornberg *et al.*, 1992; Haimovich *et al.*, 1993). FAK is a multifunctional tyrosine kinase, known to interact

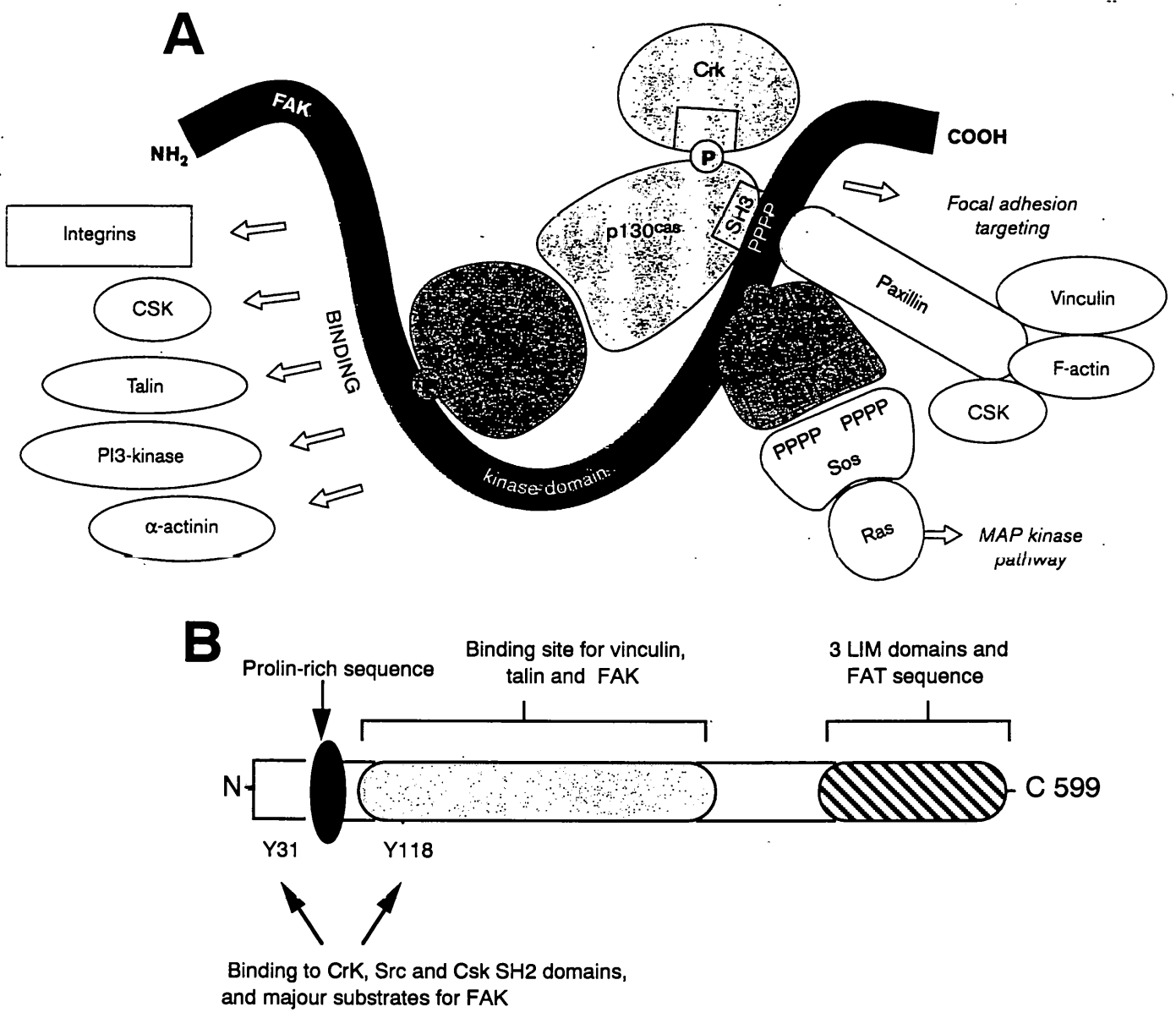


Figure 1.8 Structural features and molecular interactions of FAK and paxillin.

Panel A: FAK is a multifunctional tyrosine kinase that binds to a remarkable number of cytoskeletal and signalling molecules. Src family kinases bind to one phosphotyrosine site (P) of FAK and mediate the phosphorylation of a second site required for binding of Grb2. PPPP, proline rich sequence; NH_2, amino terminus; COOH, carboxyl terminus; CSK, C-terminal Src kinase; PI3-kinase, phosphatidylinositol 3'-kinase. (From Yamada and Geiger, 1997).

Panel B: Structural features of paxillin. FAT (focal adhesion targeting) sequence; Csk, C-terminal Src kinase. N and C indicate N-and C-terminal, respectively, with the residues number indicated.

with several proteins *in vitro* (Fig. 1.8A). Paxillin, another well characterised component of focal adhesions, is known to be phosphorylated by FAK *in vitro* , together with tensin (Turner *et al.*, 1990; Bellis *et al.*, 1995)(Fig. 1.8B). Moreover, tyrosine phosphorylation of paxillin parallels the activation of FAK during early embryonic development (Turner *et al.*, 1991; Turner *et al.*, 1993), upon integrin-mediated cell adhesion or antibody-induced receptor clustering (Kornberg *et al.*, 1991; Burridge *et al.*, 1992), and in response to a variety of mitogens (Zachary *et al.*, 1992; Zachary *et al.*, 1993; Rankin and Rozengurt, 1994). These evidences suggest that the activity of FAK and paxillin tyrosine phosphorylation may be closely related, although their exact role in focal adhesion assembly and cytoskeletal reorganisation is not clear yet. A possible mechanism of action of tyrosine phosphorylation in focal adhesions involves the binding of SH2 domains present in many signalling molecules and some cytoskeletal molecules to protein tyrosine phosphorylated sites (Pawson, 1995). This provides a mechanism for assembling protein complexes which can be readily regulated by a host of protein tyrosine kinases and phosphateses. Paxillin is believed to play a role in the assembly of focal adhesions, by forming complexes with several components of the adhesive sites, as shown by *in vitro* and *in vivo* studies (Birge *et al*, 1993; Hildebrand *et al.*, 1995; Sabe *et al.*, 1994; Turner and Miller, 1994; Weng *et al.*, 1993). *In vitro* studies have shown that the SH2 domains of Crk, Csk, Src and Fyn can recognise tyrosine phosphorylated paxillin (Schaller and Parson, 1995), but still the role of paxillin tyrosine phosphorylation *in vivo* remains to be estabilished. Moreover, paxillin contains 3 LIM domains at the C-terminus (Fig. 1.8B). These are cysteine and histidine rich sequences that are organised in Zn-fingers and appear to be responsible in mediating protein-protein interactions (Gill, 1995), and they represent the principal determinant of paxillin localisation to focal adhesions (Brown *et al.*, 1996). Several evidences suggest that tyrosine phosphorylation of FAK and paxillin may play a role in focal adhesion assembly, including the finding that tyrosine kinase inhibitors block the assemby of both stress fibers and focal adhesions, accompanied by a decrease of tyrosine phosphorylation of FAK and paxillin (Burridge *et al.*, 1992; Defilippi *et al.*, 1994). However, the fact that FAK-/- cells are still able to form focal adhesions (Ilic *et al.*, 1995), make these conclusions controversial, and raises the possibility that another tyrosine kinase may play a major role in focal adhesion assembly. Interestingly, several FAK isoforms are produced from internal promoter within the gene and through alternative splicing. For instance, chicken cells express an N-terminally truncated form of FAK, termed FRNK, which probably acts as a negative regulator of FAK activity (Richardson and Parson, 1996). Moreover, a FAK-

related protein, called proline-rich tyrosine kinase 2 (Pyk2), is highly expressed in the central nervous system and in cells of the haematopoietic lineage (Girault *et al.*, 1999; Avraham and Avraham, 1997)

Although tyrosine phosphorylation has been better characterised, other types of phosphorylation are also likely to play significant roles in the formation of adhesive complexes. For example, protein kinase C has been implicated in the formation of focal adhesions (Woods and Couchman, 1992). Serine phosphorylation of paxillin was observed after adhesion of macrophages to vitronectin, via the integrin αVβ5 in a process that was independent of FAK and apparently mediated by protein kinase C (De Nichilo and Yamada, 1996), and after adhesion of CHO cells to fibronectin (Brown *et al.*, 1998). Whether serine phosphorylation of paxillin may play a role in the assembly or regulation of focal adhesion is still unclear.

1.4 THE ACTIN CYTOSKELETON

1.4.1 The regulation of actin polymerisation

Actin filaments consist of two double tight helices of uniformly oriented actin molecules (globular, G-actin). Polymerisation of pure actin *in vitro* requires ATP as well as divalent cations, usually Mg^{2+}, and occurs with an initial lag phase, corresponding to nucleation of the first three actin monomers, and a rapid elongation phase (Fig. 1.9). Actin filaments are polarised, presenting a slow-growing minus end, and a fast-growing plus end. Because myosin heads lean to confer an arrowhead structure on actin filaments visible in the electron microscope, the minus end is also referred to as the "pointed end", and the plus end as the "barbed end". The polymerisation rate is different at the two ends of the actin filament: the plus (or barbed) end polymerises at up to 10 times the rate of the minus (or pointed) end. The critical concentration for actin polymerisation *in vitro*, that is the free monomer G-actin concentration required for actin polymerisation, is 0.2 μM. Shortly after polymerisation, the terminal phosphate of the ATP bound to the actin molecule is hydrolysed to ADP. Hydrolysis serves to weaken the bounds in the polymer and thereby promote depolymerisation.

Actin filaments and monomeric G-actin are maintained in a dynamic steady state. As a result of the dissipative nature of the polymerisation reaction, the flux of ATP-G-

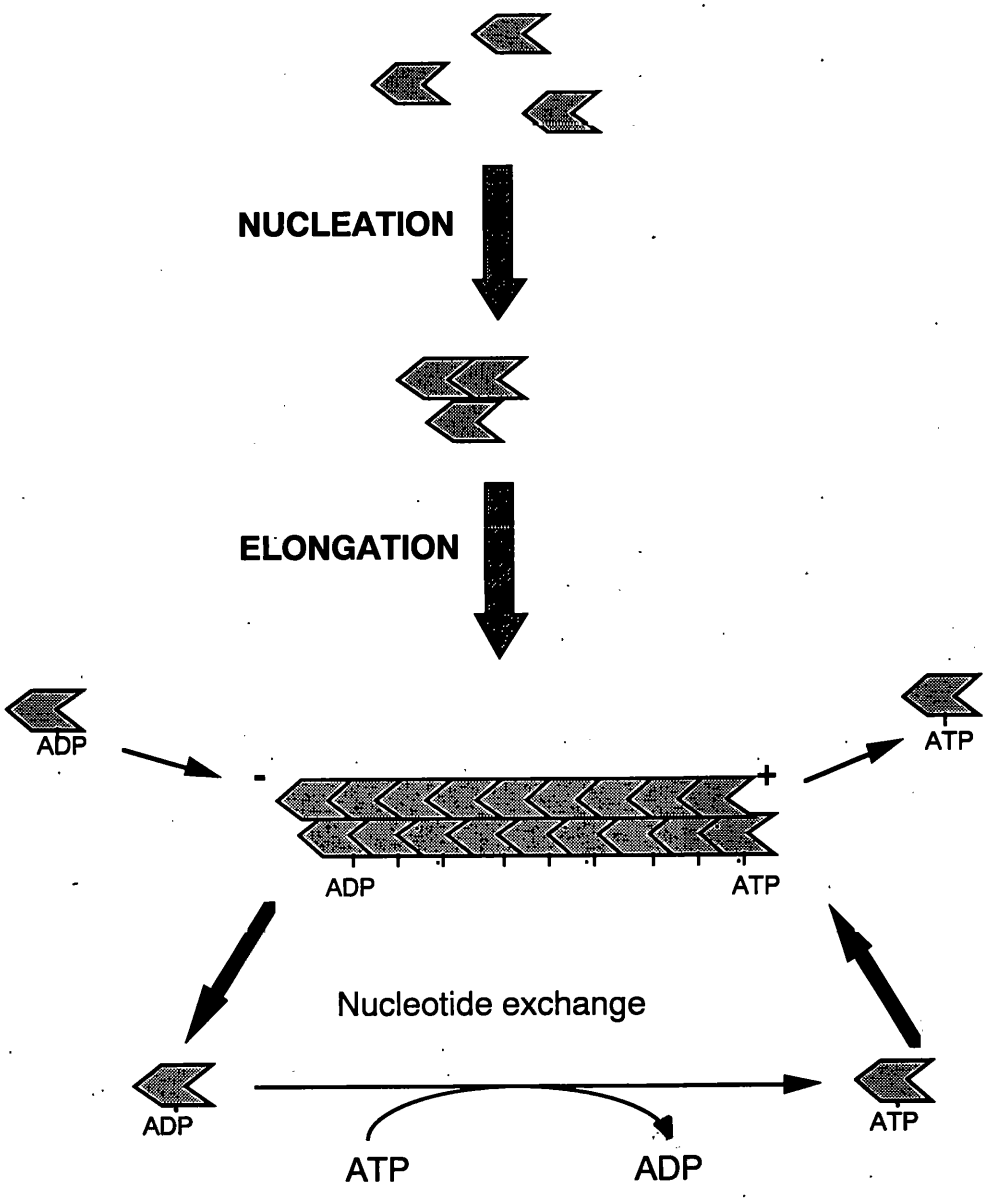


Figure 1.9 Schema of actin polymerisation *in vitro*.

In vitro actin polymerisation occurs through a slow nucleation phase, and an elongation phase. Polymerisation at the plus end (+) is favoured compared to depolymerisation, which occur preferentially at the minus (-) end. Shortly after polymerisation, ATP on actin is hydrolysed to ADP. ADP-G-actin is reloaded with ATP by nucleotide exchange.

actin association into barbed ends is balanced by the dissociation flux of ADP-G-actin subunits from the pointed ends, resulting in what is known as a treadmilling process (Fig. 1.9). Although much is known about actin polymerisation *in vitro*, the situation is very different in the cell. In fact, within cells monomeric actin is present at a concentration well above the one required for rapid polymerisation *in vitro*, suggesting that the cell has evolved special mechanisms for the control of actin polymerisation. Moreover, actin filaments can be organised in a wide range of different structures, ranging from well organised and compact stress fibers, where bundles of actin filaments are kept together by cross-linking proteins, to more dynamic structures, such as those present in lamellipodia or filopodia. More than 200 actin-binding proteins are known in vertebrates, potentially involved in the modulation of actin properties and functions, indicating that the mechanisms underneath regulation of actin dynamics are very complex.

One way that cells control actin assembly is by sequestering monomeric actin through binding to proteins such as tymosin β4, which is the most abundant G-actin binding protein in cells, and profilin, which is present in all cells and is thought to play a part in controlling actin polymerisation in response to extracellular stimuli. Profilin accelerates the exchange of ATP for ADP on actin monomers, thus favouring the polymerisation from the barbed end. Cells also contain a variety of barbed ends capping proteins. Gelsolin and its relatives (severin, adseverin/scinderin, villin and fragmin) bind very tightly to barbed ends and sever filaments in response to certain stimuli, such as the increase in the intracellular concentration of PIP2 and calcium (Weber *et al.*, 1991). Other "weaker", non-severing capping proteins (which bind actin with a lower affinity) have recently received much attention. These are capping protein β2, which is the homologous of Cap Z in non-muscle cells, and Cap G (Schafer and Cooper, 1995).

The rate of polymerisation, especially at the pointed end, is controlled preferentially by ADF (Actin depolymerizing factor)/cofilin proteins. These are ubiquitous, conserved actin binding proteins binding preferentially to the ADP-bound forms of G-actin and F-actin. They increase by 25 folds the rate of depolymerisation of ADP-F-actin specifically from the pointed ends. (Carlier *et al.*, 1997).

1.4.2 The problem of actin nucleation in the cell

The rate limiting step in actin filament formation involves the generation of an assembly-competent actin nucleus, such as an actin trimer. Mechanisms that nucleate actin filaments in living cells have not been elucidated yet, but the fact that adhesion to the

extracellular matrix can stimulate vectorial cell migration suggests that spatial coincidence must exist between sites of substratum recognition by integrins and the actin machinery necessary for cell surface extension. Some insights in this mechanism come from the movement of intracellular pathogens such as *Listeria*, and from genetic studies of budding yeast. *Listeria* uses a single protein (ActA) to initiate actin assembly and to move inside the host cell (Higley and Way, 1997). The central domain of ActA contains a proline-rich repeat which forms the binding site for VASP (vasodilatator-stimulated phosphoprotein) (Chakraborty *et al.*, 1995). VASP belongs to the Enabled/mammalian Enabled (Mena)/VASP family of proteins, targeted to proline-rich ligands in focal adhesions (possibly to zyxin or vinculin, see below) via their EVH1 (Enabled/VASP homology 1) domain, which is also found in WASP (Wiscott-Aldrich syndrome protein). VASP/Enabled/WASP proteins act as molecular connectors, recruiting other ligands via their prolin-rich sequence. One potential link between VASP and actin polymerisation comes from the ability of this protein to multimerise and to dock profilin through the prolin-rich sequence (Kang *et al.*, 1997).

Interestingly, a proline repeat similar to those found in ActA is present in the focal adhesion protein vinculin and binds to VASP (Brindle *et al.*, 1996); however, the ability of vinculin to cooperate with VASP to regulate actin assembly has not been proven yet. On the other hand, several approaches including biochemical, immunological, and functional studies suggest that one mammalian protein mimicked by ActA is the focal adhesion component zyxin (Beckerle, 1997; Golsteyn *et al.*, 1997). The proline region of zyxin has three known partners: α-actinin, Vav and VASP. All of these proteins have themselves been implicated in actin assembly and organisation. α-actinin could serve to cross-link actin filaments and to tether these cytoskeletal elements directly to integrin receptors. Vav is a protooncogene product that interacts with zyxin via its C-terminal SH3 domain (Hobert *et al.*, 1996); it exhibits guanine nucleotide exchange activity for Rho family members (see 1.4.3)(Olson *et al.*, 1996; Crespo *et al.*, 1997), and thus may contribute significantly to zyxin's ability to stimulate changes in the actin cytoskeleton. Similarities between actin assembly on the surface of Listeria and at the cytoplasmic face of the plasma membrane is shown in Fig 1.10. In searching for cellular factors that initiate actin assembly at the surface of *Listeria*, Welch *et al.* (1997) isolated a stable complex containing the actin-related proteins Arp2-Arp3, which is highly conserved among eukaryotes. At least two factors acting sequentially to nucleate actin assembly have been isolated in yeast, namely Bee-1, and pca-1 (Lechler and Li, 1997). Interestingly, Bee-1 is a yeast homolog of the members of the VASP/Enabled/WASP

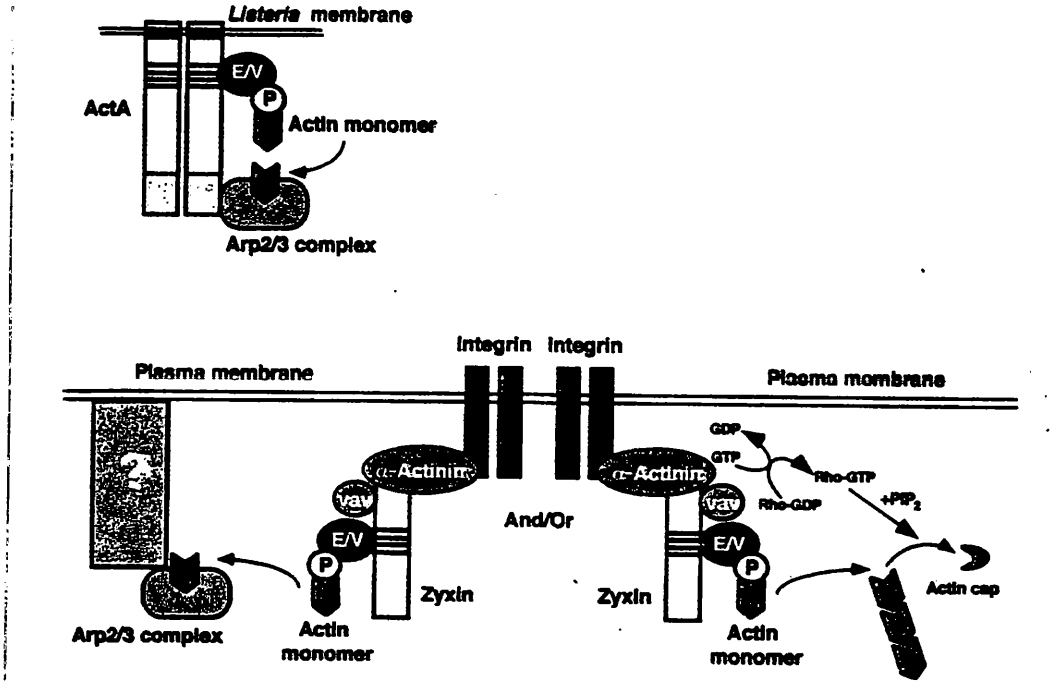


Figure 1.10 Similarities between actin assembly on the *Listeria* surface and at the cytoplasmic face of the plasma membrane.

The *Listeria* ActA protein appears to stimulate actin assembly by interacting with the Arp2/3 complex and members of the Ena/VASP family (E/V) that recruit profilin (P). In an uninfected mammalian cell, one region of zyxin may function like ActA recruiting Ena/VASP and ultimately profilin. Zyxin also binds α-actinin, which could facilitate the cross-linking of newly assembled actin filaments as well as linking zyxin directly to integrin receptors. Vav bound to zyxin may locally reduce actin filaments capping, thus enhancing filaments elongation. From Beckerle, 1998.

family (Li, 1997), suggesting that the mechanism for the nucleation of actin filaments is conserved from yeast to mammals.

1.4.3 Role of Rho GTPases in the reorganisation of the actin cytoskeleton

The Rho family of small GTP-binding proteins, including Rho, Rac, and Cdc42, has attracted much attention in the last years for their role in many cellular functions, including the regulation of the actin cytoskeleton and of focal adhesion organisation. Rho family proteins act as molecular switches cycling between an active, or GTP-bound state, and an inactive, or GDP-bound state (Fig. 1.11A). In their GTP-bound form they associate to the membrane via a post-traslational modification at their C-terminal. In general, these proteins have a low intrinsic GTPase activity. Cycling of nucleotides is regulated by several different molecules, including guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDI)(Fig. 1.11A).

The role of Rho GTPases in the regulation of the actin cytoskeleton is best characterised in fibroblasts, where several studies have described distinct types of organisation of the actin cytoskeleton following activation of different Rho members. In Swiss 3T3 fibroblasts, RhoA induces the organisation of contractile actin-based stress fibers; Rac proteins are involved in the formation of dynamic actin structures at the cell periphery, such as membrane ruffles and lamellipodia; and Cdc42 has been implicated in the extension of actin-rich protrusions, called filopodia (Fig. 1.11B); in addition, all three proteins are required for focal adhesion assembly (Kozma *et al.*, 1995; Ridley *et al.*, 1992; Nobles and Hall, 1995). It has been demonstrated that Rac is able to activate Rho (Ridley *et al.*, 1992) and that a cascade of activation occurs in the cell: Cdc42 activates Rac, which in turn activates Rho; still each protein maintains a specific and independent effect.

Several potential downstream targets for Rho proteins have been identified, including protein kinases and adaptor proteins (Van Aelst and D'Souza-Schorey, 1997). The assembly of actin stress fibers by Rho is mediated, at least in part, by the activation of a serine/threonine kinase (ROKα), which phosphorylates and inhibits the activity of myosin light chain phosphatase, resulting in an increased levels of myosin light chain phosphorylation (Kimura *et al.*, 1996). This favours the formation of an acto-myosin complex, and promotes an actin-activated myosin ATPase activity (Burridge *et al.*,

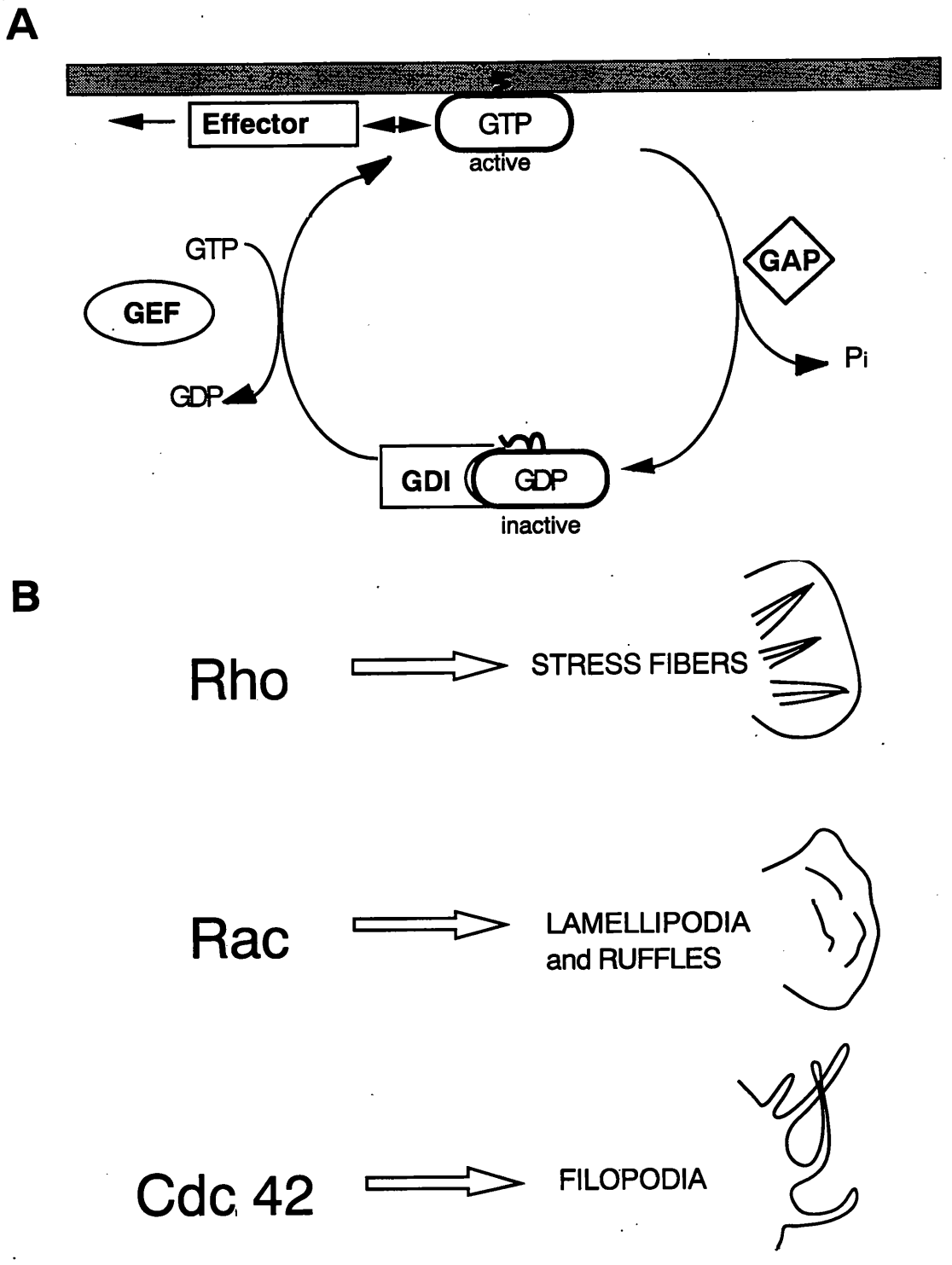


Figure 1.11 The cycle of the Rho family GTPase and their function in fibroblasts.

Panel A: The Rho family GTPases are known to cycle between an active, or GTP-bound state, and an inactive, or GDP-bound state. The cycle is regulated by Guanine nucleotide Exchange Factors (GEF), GTPase Activating Proteins (GAP), and Guanine nucleotide Dissociation Inhibitors (GDI).
Panel B: The function of the GTPases Rho, Rac and Cdc42 in the reorganisation of the actin cytoskeleton in fibroblasts.

1996). Contraction of acto-myosin is thought to lead to the alignment of actin filaments into bundles. Moreover, evidence has been presented that this results in the clustering of integrins and of associated cytoskeletal proteins in the plane of the plasma membrane to form focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996). Rho is known to activate also the phosphatidylinositol (PI)-5-kinase resulting in an elevation of PIP_2 levels (Chong *et al.*, 1994). Several G-actin binding proteins (e.g. profilin, see 1.4.1) are induced to release actin upon interaction with PIP2. Similarly, proteins that cap the ends of actin filaments, such as gelsolin (see 1.4.1), dissociate from filament ends in response to PIP_2. Together, these effects of PIP_2 promote actin polymerisation. In addition, elevated PIP_2 enhances the interaction of α-actinin with actin filaments, and of vinculin with talin and actin (as described in 1.3.3), thus promoting both bundling and attachment of actin filaments to integrins, and contributing to the assembly of focal adhesions.

The role of Rac and Cdc42 in the reorganisation of the actin cytoskeleton is less well characterised. One downstream target of both Rac and Cdc42 is the serine/threonine kinase PAK (Manser *et al.*, 1994). This kinase is implicated in the regulation of actin organisation and focal adhesion assembly (Sells *et al.*, 1997), but its molecular mechanism of action is controversial. Rac and Cdc42 are also known to increase the level of PIP2, resulting in the uncapping of actin filaments barbed ends (Hartwig *et al.*, 1995). Cdc42 can also bind to WASP (Aspenstrom *et al.*, 1996)(see 1.4.2). Moreover, Rac has been recently shown to regulate the phosphorylation of the myosin II heavy chain inducing the disassembly of acto-myosin complexes (van Leeuwen *et al.*, 1999). This suggests an antagonistic mechanism to Rho function, and adds a new function to Rac in regulating the actin cytoskeleton.

1.5 AIM OF THE WORK

It is now becoming evident that focal adhesions are not just static anchoring structures, but they are rapidly and dynamically regulated during the process of adhesion, and during important functions such as migration. Most of the information that we have about focal adhesion sites have been obtained from colocalisation studies and from *in vitro* reconstitution of the interactions between purified components. Still the sequence of molecular events leading to focal adhesions organisation, the way they are regulated, and the exact role of the different focal adhesion components have not been clarified yet.

Much attempts have been made in the last years in this direction, but still the information that we have is largely fragmentary. This is probably due to the complexity of the crosstalk between integrins and other signalling pathways occurring in the cell at the same time. The complexity is further increased by peculiarities that may be present in the different cell types studied.

We think that a "cell-free" system would make an important contribution in the field, allowing the dissection, and possibly the reconstitution, of some of the molecular events leading to focal adhesion organisation and regulation, in an environment at least partially separated from other signalling pathways occurring in the intact cell. Successfull attempts have already been made in this direction. They include studies in which receptor-stimulated actin polymerisation has been achieved in permeabilised neutrophils (Redmond *et al.*, 1994) and platelets (Hartwig *et al.*, 1995). Furthermore, permeabilised Swiss 3T3 cells have been utilised to show the involvement of activated RhoA GTP-binding protein in the stimulation of phosphorylation of $p125^{FAK}$ and paxillin (Seckl *et al.*, 1995). Crowley and Horwitz (1995) have used permeabilised chicken fibroblasts to show an ATP-dependent destabilisation of focal adhesions during cell detachment. McKay *et al.* (1997) have shown that moesin, ezrin and radixin can reconstitute actin polymerisation and focal complex formation in response to activation of Rho and Rac in serum starved Swiss 3T3 cells permeabilised with digitonin. More recently, Norman *et al*. (1998) have demonstrated an Arf1-mediated recruitment of paxillin to focal adhesions in permeabilised Swiss 3T3 fibroblasts.

In this work, I will use a modification of the lysis-squirting technique (Avnur and Geiger, 1981; Nermut *et al.*, 1991), for the preparation of detergent-free ventral plasma membranes (VPMs) obtained from adherent chicken embryo fibroblasts (CEFs). Two important advantages of this system make it suitable as a "cell-free" system for studies on focal adhesion organisation: the maintenance of the adhesive receptors within their natural lipidic environment (i.e. the adherent portion of the plasma membrane of cells spread on ECM), and the accessibility to the cytoplasmic side of the adhesive membrane, without need for detergents that may affect the environment of the adhesive receptors.

The first part of my work will deal with the morphological and biochemical characterisation of VPMs. Together with giving the basis for the functional studies of the second part of the thesis, this work will allow to obtain new important information concerning the molecular composition of focal adhesion sites. In order to obtain new important tools for functional studies about focal adhesion regulation, I will describe the production and characterisation of some mAbs raised against VPMs preparations.

In the second part of my work, I will describe the use of VPMs as a "cell-free" system to obtain new insights in the mechanism of organisation and regulation of the adhesive sites and of the connected actin cytoskeleton. It is known that the strength of adhesion should be finely regulated in order to allow a cell to move and to perform several important functions, and this regulation could act at the level of integrin affinity modulation for the extracellular ligands; how the cell can sense extracellular signals, and the molecular mechanism underneath these events are not clear yet. By using VPMs as a "cell-free" system, I will set up the conditions for the modulation of the function and redistribution on the membrane of integrin receptors. The requirements of various focal adhesion components and of the connected actin cytoskeleton will also be investigated.

The described "cell-free" system will be used also for the reconstitution of some of the interactions occurring at the adhesive sites, in particular between β1 integrin receptors and α-actinin. Reconstitution of adhesive complexes from purified components is made difficult by the fact that focal adhesion assembly occurs at the plasma membrane where clusters of receptors may be necessary, and both extracellular ligand and intracellular milieu may be required. Reconstitution of the binding of focal adhesion proteins to permeabilised or partially disrupted cells has been previously reported (Avnur *et al.*, 1983; Ball *et al.*, 1986). One important advantage of our "cell-free" system is that we can test the binding of purified components to VPMs where integrin function can be modulated.

Focal adhesions are postulated to act as sites of signalling for the regulation of the connected actin cytoskeleton, and this event has a great biological importance in several aspects of cell behaviour. In order to start investigating the relationship between focal adhesions and the actin cytoskeleton, I will describe the reconstitution of the recruitment of exogenous actin on VPMs, and study the way it can be regulated by the presence of the focal adhesion complex.

- *Chapter 2* -

MATERIALS AND METHODS

2.1 CELL CULTURE

Chicken Embryo fibroblasts (CEFs) were isolated from 10-day-old embryos. The head, legs, wings and internal organs were removed and the embryo was squirted through a 10 ml syringe without a needle and trypsinized with 5 ml 0.1 trypsin in PBS for 15 min at room temperature. The suspension above the debris was taken and 10 ml of complete medium (DMEM containing 5% fetal calf serum (FCS), 1% chicken serum, 100 U/ml penicillin and streptomicin, 20 mM glutamine) was added. After a 5 min spin at 500 g, the pellet containing cells was resuspended in 10 ml complete medium, filtered through a nylon membrane and the cells were counted. 0.5-2.0 x 10^6 cells were plated on each 100 mm diameter petri dish at 37°C, 5% CO_2. Each dish reached confluence in 2-3 days. A confluent dish of CEFs was washed twice with 10 ml of phosphate-buffered saline (PBS) and trypsinized by approximately 2 min shaking with 1 ml 0.02 % trypsin in PBS at room temperature. The trypsin was bloked by addition of 9 ml of complete medium, cells were resuspended and plated at a dilution of 1:5-1:10. After the second passage, CEFs were frozen and conserved in liquid nitrogen in 1 ml aliquots containing 0.5-2.0 x 10^6 cells in 50% FCS, 40% complete medium and 10% DMSO. When used, each aliquot was thawed and added to 10 ml of complete medium, and plated in a 100-mm diameter culture dish which reached confluence in 2-3 days. CEFs up to the fifth passage were used for experiments.

2.2 ANTIBODIES

The production, purification and use of the pAb β1-cyto raised against a peptide corresponding to the last 23 amino acids from the C-terminal cytoplasmic domain of β1 (Tomaselli *et al.*, 1988), and of the pAb α6–EX against the amino-terminal portion of the chicken α6 subunit (de Curtis and Reichardt, 1993), have been previously described. The mAb CSAT against the chick integrin β1 subunit (Neff *et al.*, 1991) was a generous

gift from Dr. A. F. Horwitz (University of Illinois, Urbana, Illinois). The production and characterisation of the mAb TASC against the chick integrin β1 subunit has been previously described (Neugebauer and Reichard, 1991; Shih *et al.*, 1993). The TS2/16 anti-human β1 integrin mAb was a generous gift of Dr. G.Tarone (University of Torino, Italy). The mAbs against actin, vinculin, tubulin, tensin and talin were purchased from Sigma Chemical Co (St. Luis, MO). The mAb against paxillin was from Zymed Laboratories, Inc. (San Francisco, CA). The pAb recognising FAK was a kind gift of Dr. G. Tarone, University of Torino, Italy, while the mAb 2A7 against FAK was from Upstate Biotechnology Inc. (Lake Placid, NY). The pAb AAL20 against actin was a generous gift from Dr. C. Chaponnier (University of Geneva, Switzerland), while the rabbit pAb anti-ezrin was raised against the entire ezrin produced in bacteria, as previuosly described (Algain *et al.*, 1993), and was a gift from Dr. T. Crepaldi (University of Torino, Italy). MAbs PY20 (Glenney et al., 1988) and PT66 recognising tyrosine-phosphorilated polypeptides were purchased from ICN (Costa Mesa, CA) and Sigma Chemical Co (St Luis, MO). The pAb against Rac 1 was raised against recombinant Rac 1 and was a generous gift from Dr. A. Hall (Medical Research Concil Laboratory of Molecular Cell Biology, University College London, UK). The mAbs M2D5 (IgG) and X1E8 (IgM) were obtained by injecting VPM preparations into mice and by subsequent screening of the hybridoma clones by immunofluorescence, as described in the corresponding pharagraph.

2.3 PREPARATION OF VENTRAL PLASMA MEMBRANES (VPMs)

A confluent dish of CEFs was washed twice with PBS and trypsinized as described in 2.1. The cells were pelleted by 5 min centrifugation at 500 g and washed twice with 10 ml of serum-free medium. 1/60 to 1/20 of the resuspended cells were added to 15 mm or 13 mm diameter coverslips, or to a 100-mm culture dish. Glass coverslips were cleaned by boiling in 0.1 M HCl, washing with 70% ethanol, rinsing with double distilled water, and drying in air. When indicated, coverslips were previously coated with 20 μg/ml fibronectin. For experiments with VPMs on laminin, coverslips were treaated with dimetylclorosilane in trichloroethane (BDH Laboratories supplies, England) before incubation with 100 μg/ml laminin for 18 h at room temperature, and blocking of non specific binding by incubation with 1% Bovine Serum

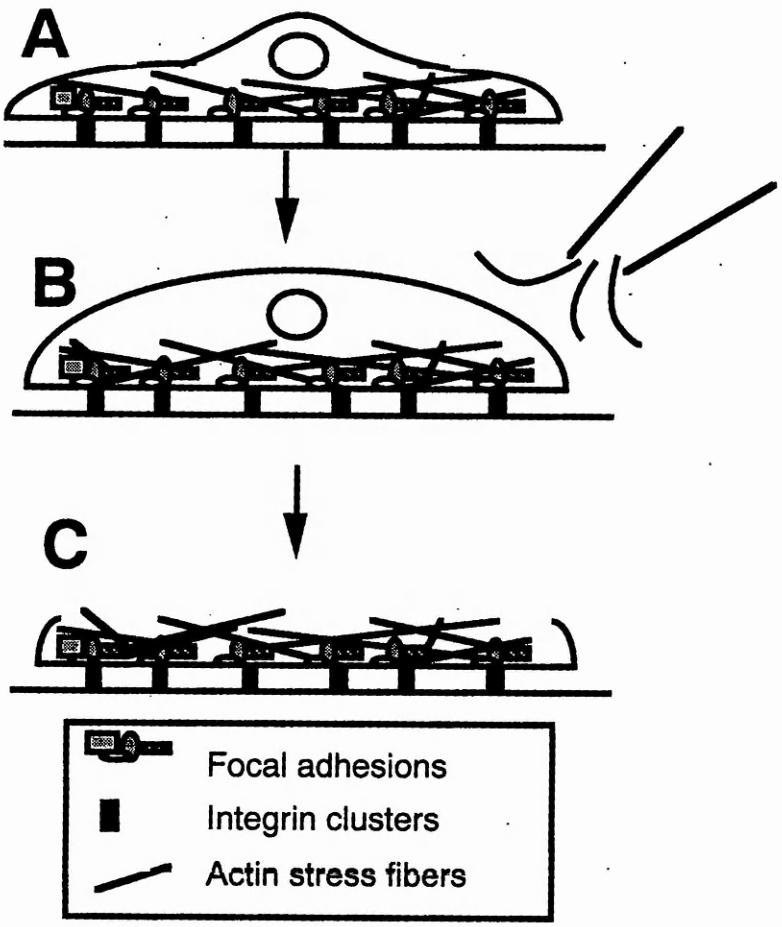


Figure 2.1. Preparation of Ventral Plasma Membranes (VPMs).

Panel A: CEFs were cultured and allowed to adhere for 18h on the extracellular matrix. Panel B: Adherent cells were transferred on ice, washed with an hypotonic buffer and squirted over under a jet of the same buffer. Panel C: Ventral membrane of cells remained attached to the substrate, together with clusters of integrin receptors in connection with the actin cytoskeleton.

Albumin (BSA) in PBS for 2 h at 37°C. In this case, CEFs were cultured overnight in the absence of serum, and allowed to adhere on laminin-coated coverslips for 3 h in serum-free medium, in the presence of 25 μg/ml cycloheximide, to avoid extra cellular matrix (ECM) production. The volume of the medium was adjusted to 2 ml for the 15 mm, 1 ml for the 13 mm, and to 10 ml for the 100-mm diameter culture dishes. VPMs from cells on coverslips cultured overnight (or for 3 h, when indicated) in serum-free medium were prepared utilising a modification of the lysis-squirting technique (Nermut *et al.*, 1991). Cells were washed twice with ice-cold 20mM Hepes-KOH buffer, pH7. After 1 minute, cells were squirted over by using a jet of the same ice-cold buffer from a water bottle, and immediately fixed with 3% paraformaldehyde or processed for the "cell-free" assay, as described below (see figure 1). For biochemical analysis, VPMs were prepared by the same procedure, but in this case the jet of buffer (20 mM Hepes-KOH pH 7, 0.3 mM PMSF) was produced by forcing it under air pressure through a pipette.

2.4 "CELL-FREE" ASSAY

VPMs prepared from cells grown on 13 mm diameter glass coverslips or on 100-mm diameter culture dishes were incubated for the indicated time at 37°C in a humid chamber in 25 μl or 10 ml respectively, of low calcium buffer (LCB, containing 125 mM K-acetate, 2.5 mM $MgCl_2$, 12 mM glucose, 25 mM Hepes-KOH, pH 7 and 50 nM free Ca^{2+}), or high calcium buffer (HCB, containing 125 mM K-acetate, 2.5 mM $MgCl_2$, 12 mM glucose, 25 mM Hepes-KOH, pH 7 and 1 mM free Ca^{2+}). EGTA-$CaCl_2$ buffer were used to obtain defined [Ca^{2+}], according to published procedures (Bers *et al.*, 1994). Buffering conditions different from those specified here have been described, when used, in Chapter 3. For stress fiber disassembly, VPMs were incubated for 3 min at 0°C in HCB containing 4 μM fusion protein corresponding to the full length gelsolin (Way *et al.*, 1989), a generous gift from M.Way (European Molecular Biology Laboratory, Heidelberg, Germany). After incubation at 37°C, VPMs on coverslips were lifted with 200 μl of incubation buffer, and immediately transferred in 3% paraformaldehyde to be processed for immunofluorescence. VPMs in petri dishes were transferred on ice, washed twice with 10 ml incubation buffer and lysed for biochemical analysis. Untreated VPMs kept at 0°C were used as controls for immunofluorescence and for biochemical analysis. In some experiments, CEFs were treated in extracellular

buffer (20 mM Hepes, 2.7 mM KCl, 137 mM NaCl, 5.6 mM glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH7) before VPMs preparation.

2.5 BIOCHEMICAL METHODS

2.5.1 Preparation of lysates from CEFs and VPMs

VPMs were prepared from CEFs cultured overnight in serum-free medium on a 100 mm diameter culture dish and treated for the "cell-free" assay, as described in 2.4. For each experimemt, two dishes of VPMs or one of confluent CEFs were transferred on ice, washed twice with 10 ml of ice-cold TBS (20 mM Tris-HCl, pH7.5,150 mM NaCl, 1mM $CaCl_2$, 1 mM $MgCl_2$), solubilised with 0.25 ml of either SDS-PAGE loading buffer (Laemmli, 1970), or lysis buffer (TBS containing 1% Triton X-100, 1mM sodium orthovanadate, 10mM sodium fluoride, and 10 μg/ml each of antipain, chymostatin, leupeptin and pepstatin), and scraped with a bent syringe needle. For the preparation of lysates from embryonic chick brain, an E13 brain was dissected, transferred on ice, washed with ice-cold TBS, and solubilised with 9 volumes of lysis buffer by 6 times douncing in a potter tube. Lysates obtained from VPMs, CEFs or chick brain were then transferred to tubes, and rotated for 15 min by end-over-end mixing at 0°C. The insoluble material was removed by centrifugation at 0°C for 15 min at 12,000 g in a refrigerated centrifuge. Protein concentration was determined by using a kit from Biorad (Hercules, CA) (Bradford, 1976).

2.5.2 Immunoprecipitation

For immunoprecipitation, rabbit anti-mouse Ig and specific mAbs were preadsorbed for 2h to 20 μl Protein-A Sepharose beads (Pharmacia, Piscataway, NJ) The beads were washed 3x with 1 ml of washing buffer (lysate buffer containing only 0.2% Triton X-100), added to 100-300 μg of protein from cell or VPM lysates, and incubated for 1h at 4°C by end-over-end mixing. The beads were washed 2x with 1.5 ml of washing buffer and resuspended in SDS-PAGE loading buffer for SDS-PAGE analysis.

2.5.3 SDS-PAGE and immunoblotting

Immunoprecipitates, lysates and purified proteins were analysed by SDS-PAGE on the indicated % acrylamide gels, according to Laemmli (1970). Gels were electrophoretically transferred to 0.2 mm nitrocellulose filters and stained with Ponceau S (0.2% in 3% TCA, destain with dd-water) to visualise molecular weight standards and proteins. After blocking the nitrocellulose with 50 mM Tris-Cl, 150 mM NaCl , 5% non-fat dry milk , pH 7.5, for 1h at room temperature, filters were incubated overnight at 4°C in the same buffer containing the primary antibodies at the following dilutions: anti-phosphotyrosine antibodies, 2 μg/ml PY20 plus PT66 ascites 1:1000; anti-paxillin antibody, 1 μg/ml purified IgG; anti-actin, 1:2000 ascites; anti-talin, 1:100 ascites; anti-vinculin, 1:200 ascites; anti-tensin 1 μg/ml purified IgG; anti-β1 integrin subunit, 6 μg/ml purified IgG; anti-α6 integrin subunit, 9 μg/ml purified IgG; anti-tubulin, 1:1400. MAbs and pAbs were detected by 1h incubation at room temperature with 0.2 μCi/ml of ^{125}I-sheep anti-mouse IgG or ^{125}I-Protein A, respectively (Amersham, Aarlington Height, IL). Filters were exposed to Amersham Hyperfilm-MP. Quantification was done from autoradiograms by using a computing densitometer from Molecular Dynamics (Sunnyvale, CA).

When necessary, filters were stripped according to Cooke et al., 1991: filters were incubated by shaking for 1 h at room temperature in denaturation buffer (7 M Guanidinium-HCl, 50 mM Tris-HCl, 2 mM EDTA, 50 mM DTT, 10 mM Phenylphosphate, pH 7.5); proteins on filters were than renaturated by 12-18 h incubation at 4°C with shaking in renaturing buffer (50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, 2 mM DTT, 0.1% NP-40, pH 7.5), and washed 4 times with 50 mM Tris-HCl, 150 mM NaCl, pH 7.5. Filters were dried and exposed to check the success of the stripping procedure, before being re-probed with different primary antibodies.

Based on the densitometric values obtained from the blots, the percentage of recovery of polypeptides in the VPM fraction was calculated as the percentage of polypeptides recovered in VPM lysate from one 100 mm culture dish, compared to the amount of the same polypeptides obtained in the lysate of one 100 mm culture dish of intact CEFs. The amount of polypeptide per milligram of protein in the fraction was defined as relative concentration. Enrichments were calculated as the ratio between the relative concentration of the polypeptide in the VPM fraction, and the relative concentration in total CEF lysate, which was normalized to 1.

2.5.4 Silver staining

For silver staining, gels were fixed in 30% MetOH, 10% TCA, 10% Acetic acid for at least 2 h. They were then incubated 15 min in 50% MetOH, 12% TCA, 1% $CuCl_2$, and washed 2x10 min in 10% EtOH, 5% Acetic. After 15 min incubation in 0.01% $KMnO_4$, 2x15 min washes in 10% EtOH, 5% Acetic acid, and an extensive wash with water, 0.1% $AgNO_3$ was added for 10 min. Gels were then briefly washed with water and incubated for 1 min with 10% K_2CO3. Protein bands were revealed untill necessary with 0.0075% HCOH, 2% K_2CO3, and the reaction was stopped by adding a solution containing 10% ethanol and 5% acetic acid. Stained gels were dried at 50-60°C.

2.6 TRANSFECTIONS

The plasmid coding for the β1TR construct corresponding to the human integrin β1 subunit missing the cytoplasmic domain (Retta, *et al.*, 1998) was kindly provided by Dr. G. Tarone (University of Torino, Italy). CEFs were plated on glass coverslips in 1.5 cm diameter wells and cultured 18 h before transfection. Subconfluent cells were transfected with liposomal transfection reagent Dosper (Boehringer, Mannheim, Germany), using 3 μg of plasmid and 6 μg of Dosper/well. The Dosper and the DNA were diluted separately to a final volume of 25 μl each with 20 mM Hepes, 150 mM NaCl, pH 7.4. The Dosper was added to the DNA drop by drop, and incubated 15 min at room temperature before being added to the well containing 1 ml of fresh medium with 5% FCS. After 6 h, cells were washed and cultured in fresh medium for an additional 14 h.

2.7 RECONSITUTION EXPERIMENTS

2.7.1 Reconstitution of α-actinin binding to VPMs

Purified α-actinin was a generous gift of Dr. D.R. Critchley (University of Leicester, Leicester, United Kingdom), and was obtained from smooth muscle (chicken gizzard) as previously described (Feramisco and Burridge, 1980). For reconstitution experiments, VPMs were incubated for 10 min at 37°C in a buffer with low ionic strength (2,5 mM $MgCl_2$, 12 mM glucose, 25 mM Hepes-KOH, 5% bovine serum albumin,

pH7) with either high (1 mM) or low (50 nM) free Ca^{2+}. This was done to induce integrin redistribution. When indicated, the buffer also contained 4 μM gelsolin to remove endogenous actin. VPMs were then washed once for 2 min at room temperature with 1 ml of the same buffer (without gelsolin), and further incubated for 10 min at 37°C in 20 μl/13 mm coverslip of the same buffer. When indicated, 100 μg/ml of purified α-actinin were present during this incubation. Samples were washed for 2 min at room temperature in 1 ml of the same buffer without α-actinin, and fixed for immunofluorescence.

2.7.2 Reconstitution of actin binding and polymerisation on VPMs

Actin purified from rabbit muscle (Paardee and Spudich, 1982) was a kind gift from Dr. Tony Hyman (European Molecular Biology Laboratory, Heidelberg, Germany), and it was labelled with 5-and 6-carboxytetramethyl-rhodamine succinimidyl ester (Molecular Probes, Junction City, OR) as previously described. (Kellogg *et al.*, 1988). Two cycles of assembly-disassembly were performed. The labelled globular actin (G-actin) was stored in aliquots at –80°C in 10 mM Tris-HCl, 0.2 mM ATP, 0.2 mM $CaCl_2$, 1 mM DTT, pH 8, at a concentration of 26 μM. For reconstitution experiments, control or treated VPMs were incubated for 2 min at 37°C in LCB containing 1% BSA and 1 mM rhodamine-G-actin (Rh-actin). When indicated, the buffer also contained 100 nM cytochalasin D and 100 μg/ml DNase I.

2.8 PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST VPMs PREPARATION

2.8.1 Mice immunisation

For a single injection of two mice, VPMs were prepared from 20-30 subconfluent 100-mm diameter culture dishes of CEFs plated for 18h on 20 μl/ml purified human fibronectin (Collaborative Research, Bedford, MA), in the absence of serum. Before solubilisation, the complete rupture of the cells was controlled under a phase contrast microscope. VPMs were solubilised as described above, by using 300 μl lysis buffer/dish which was transferred from one dish to the next to keep protein concentrated in the lysate. Approximately 1.5 ml lysate was collected, precipitated for 18h at –20°C using 9 volumes of ice cold acetone, and centrifuged 30 min at 9500 rpm at 4°C. The

pellet was resuspended in 300 µl of either 100 mM Tris-HCl , pH 8.8 (for the first three injections), or Phosphate-Buffered Saline (PBS) (for the following injections), and protein concentration was determined. Approximately 200µg of protein were obtained from 20-30 dishes, and the quality of the preparation was controlled by loading 10-20 µg protein on a 7.5% acrylammide gel and by comparing the preparation with a protein lysate obtained from intact cells, after silver staining, as described above. 200 µg protein were used for a single injection of two 7 weeks old Balb/c mice, with the following scheme:

-1st injection: the protein solution was emulsionated in 1:1 volume of Complete Freund Adiuvant (CFA). Approximately 1/3 of the emulsion was injected into the foot pad and 2/3 subcutaneously;

-2nd injection: 1:1 volume CFA emulsion subcutaneously injected;

-3rd injection:: 1:1 volume CFA emulsion subcutaneously injected;
-4th-9th injections: intraperitoneal injections of proteins in PBS.

The last injection was performed 5 days before fusion.

2.8.2 Cell fusion

X63 Ag8 myeloma cells were cultured in Iscove's medium (Gibco, Life Technologies) with 10% fetal calf serum (Fetal CloneIII, Hyclone), 100 U/ml penicillin and streptomycin, 20 mM glutamine, 50 µM β-mercapto-ethanol, collected, and washed 3x20 ml with Hanks' balanced salt solution (Gibco). Lymphocytes were obtained by disgregation of the spleen from the immunised mouse through a syringe without needle, filtration through a nylon membrane, and 3x20 ml washes with Hank's solution. Lymphocytes and myeloma cells were spun together at 500 g for 5 min at a ratio of 5 lymphocytes/1 myeloma cell. 1 ml of Iscove's containing 10% dymethylsulfoxide (DMSO) and 1 gr of polyethylenglicole (PEG) were added drop by drop to the pellet at 37°C under constant agitation. 19 ml Iscove's medium were added in 5 minutes under constant agitation at 37°C, and cells were centrifuged 10 min at 250 g . The pellet was resuspended in Iscove's medium with 10% fetal calf serum (Fetal CloneIII, Hyclone), 100 U/ml penicillin and streptomycin, 20 mM glutamine, 50 µM β-mercapto ethanol, and

5% Macrophage Conditioned Medium (obtained after 48-72h stimulation of P 388 D1 cells with 10 μg/ml Lypopolysaccaride from E.Coli, Difco). Cells were plated at a concentration of 10^5-10^6 cells/ml in 96 wells dishes. After 24h culture the hybridomas were selected in the same medium containing Hypoxanthine, Aminopterin, Thymidine (HAT, Gibco).

2.8.3 Screening

The screening of the hybridoma supernatants started 8-10 days after fusion and was performed by immunofluorescence on VPMs preparations. Immunofluorescence was carried out as described below, but in this case VPMs were prepared from CEFs grown on 24x50 mm coverslips and 6-20 supernatants/coverslip were tested each time: 10 μl drops of hybridoma supernatant were disposed on a wet chamber and the coverslip was leaned on the drops, 1 mm in suspension, to avoid mixture of supernatants. As control, VPMs were co-stained with β1-cyto polyclonal antibody. In each experiment a positive control, consisting of anti-vinculin monoclonal antibody, and a negative control consisting of hybridoma medium alone, were included.

2.8.4 Subcloning and expansion

The positives hybridomas were transferred to larger wells (with a first passage in medium containing HT) and frozen in culture medium containing 20-30% fetal calf serum, and 10% DMSO. In parallel, the hybridomas were subcloned by dilution to 10 cells/ml and replated in a 96 wells dish. The supernatans from wells with growing clones were screened again, and the clones from positive cells were subcloned again until all hybridomas in the plate were positive. The clones obtained were expanded in larger wells to be frozen in culture medium containing 20-30% fetal calf serum, and 10% DMSO, and conserved in liquid nitrogen in 1 ml aliquots containing 0.5-2.0 x 10^6 cells.

2.8.5 Monoclonal antibodies class determination

The Ig class of the monoclonal antibodies was determined by ELISA. Wells from a 96-well plates were preadsorbed with 20 μg/ml of anti-μ (x IgM) or anti-γ (x IgG) IgG in PBS, the free sites on plastic were blocked with 3% Bovine Serum Albumin (BSA), and 50-150 μl/well of the hybridoma supernatants to be tested were added and incubated for 2h in the wells. The unbound material was removed by 3x250 ml washes with PBS.

Wells were incubated with 1 μg/ml Alkaline Phosphatase-conjugated Goat anti-Mouse Ig, in 3% BSA. The reaction was developed with 100 ml/well of 1 mg/ml paranitrophenylphosphate in diethanolammine buffer for 5-10 min at room temperature. The reactions were blocked by the addition of 50 ml of 3N NaOH, and the O.D. at 405 nm was measured.

2.9 IMMUNOFLUORESCENCE AND MICROSCOPY

2.9.1 Immunofluorescence

After treatment of cells or VPMs under the different experimental conditions, samples were fixed for 20 min with 3% paraformaldehyde in PBS, at room temperature. After two washes with PBS, paraformaldehyde was blocked by 10 min incubation with 2.5 mg/ml NH_4Cl. After two washes with PBS, cells and VPMs were permeabilised with 0.2% Triton X-100 in PBS for 4 min (when indicated, no permeabilisation was performed), washed again with 2xPBS, and incubated 2x3 min with 0.2% gelatine in PBS. Coverslips were then incubated for 60 min at room temperature with the following primary antibodies diluted in 0.2% gelatine in PBS: anti-paxillin antibody, 40 μg/ml; anti-vinculin, ascites 1:200; anti-α-actinin mAb, ascites 1:300; β1-cyto, 10 μg/ml IgG; anti-ezrin, affinity purified IgG, 1:50; anti-Rac 1, serum 1:100; monoclonal antibody M2D5, hybridoma supernatant 1:25. In the case of TASC and CSAT, samples were incubated for 20 min with 20 μg/ml and 30 μg/ml of the antibody respectively, before fixation; incubation was at room temperature for intact cells, and at 0°C for VPMs, unless otherwise indicated. After two washes with 0.2% gelatine in PBS, the samples were incubated for 30 min with FITC-conjugated sheep anti-rabbit IgG and TRITC-conjugated sheep anti-mouse IgG (Boehringer, Mannheim, Germany), or with FITC-conjugated donkey anti-mouse IgG and TRITC-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc., West Groove, PA). Filamentous actin (F-actin) was revealed by incubation with FITC or TRITC-conjugated phalloidin (Sigma Chemical Co, St. Luis, MO). All the secondary antibodies were diluted in 0.2% gelatine in PBS. For staining with anti-FAK and anti-phosphotyrosine antibodies, a biotin-avidin amplification method was used: fixed cells and VPMs were permeabilised with 0.3% Triton X-100, and incubated 30 min at room temperature with avidin (Avidin/Biotin blocking kit, Vector Laboratories, Burlingame, CA). The samples were incubated overnight at 4°C with 10

μg/ml of the anti-FAK monoclonal antibody 2A7, or with PY20 and PT66, 20 μg/ml each. After 90 min at room temperature with Biotin-conjugated goat anti-mouse IgG, the coverslips were incubated for 1 h with Texas red-conjugated streptoavidin. For VPMs staining with the lipophilic carbocyanine dye $DiIC_{16}$ (Molecular Probes, OR), VPMs were incubated at 0°C for 5 min with 4μg/ml $DiIC_{16}$ before fixation. All coverslips were mounted with 1 drop of Gelvatol solution (20% polyvinyl alchool, 2% propylgallate, in PBS) and observed after a few hours using a Zeiss-Axiophot microscope. When observed with the objective for Interferemce Reflection Microscopy, coverslips were mounted with 50% PBS, 50% glycerol and immobilised with few drops of nail polish.

2.9.2 Immunoelectron microscopy

After treatment of VPMs for 10 min at 37°C in LCB, they were fixed, permeabilised, and incubated with first antibodies as described in the preceding paragraph. First antibodies were: β1-cyto, 10 μg/ml IgG; mAb X1E8, hybridoma supernatant 1:2; mAb M2D5, hybridoma supernatant 1:25; anti-actin polyclonal antibody AAL20, IgG fraction 1:100. For staining with the TASC mAb, intact cells were incubated 20 min at room temperature with 20 μg/ml of purified IgG before preparation of VPMs. The secondary antibodies used were anti-rabbit IgG conjugated to 18 nm colloidal gold particles, anti-mouse IgG conjugated to 6 nm colloidal gold particles, and anti-mouse IgM conjugated to 6 nm colloidal gold particles (Jackson Immunoresearch Laboratories, Inc., West Groove, PA). The samples were post-fixed in 1% glutaraldehyde, incubated in 1% osmium, and stained with 10% uranile acetate. Samples were then dehydrated and coated with a carbon thread evaporator unit (Balzer Union, Liechtenstein). They were then floated off the coverslip with 4.8% hydrofluoric acid (Miller *et al.*, 1991), collected on copper grids, and viewed with a Hitachi H7000 transmission electron microscope (Hitachi, Tokio, Japan).

2.9.3 Scanning force microscopy

VPM preparations from CEFs grown on 15 mm diameter coverslips were viewed in phase contrast at 400X through an Axiophot microscope (Zeiss, Oberkochen, Germany) and approx. 200 μm diameter circles were scribed around selected VPMs, using an eccentric diamond scribe inserted in the microscope nosepiece. Marked coverslips were mounted for examination in a NanoScope III MultiMode Scanning Force

Miscroscope (Digital Instruments, Santa Barbara, CA) resting in a vibration-damped, optical microscope (Nikon, Japan). 80-200X magnification permitted positioning of a force-sensing, cantilever stylus within a scribed circle. Pyramidal, silicon nitride styli integrated in cantilevers with spring constants of approx. 0.06 N/m (Digital Instruments specifications) were used to trace in continuous contact mode the topography of the VPM in PBS. The repulsive, contact force was typically 0.3 nN. The feedback gains ranged from 0.3 to 5 at scan speeds of approx. 500 μm/s at low magnification or 15-40 μm/s at high magnification. Relative separation of the surface and the stylus, constant force or height mode, was recorded along with the cantilever deflection error, at higher magnification.

- *Chapter 3* -

RESULTS

3.1 THE MORPHOLOGY OF VPMs

Focal adhesions are sites for integrin-mediated attachment of cultured cells to the extracellular matrix. They are formed by large clusters of integrins and by a large number of intracellular proteins which accumulate at these sites. Although a large number of polypeptides have been identified at focal adhesions, the exact architecture of these sites of tight adhesion is not known. With the aim of investigating the structure of focal adhesions and the molecular mechanism of their regulation, we have used a modification of the lysis-squirting technique (Nermut *et al.*, 1991; Cattelino *et al.*, 1995) for the preparation of detergent-free VPMs obtained from adherent chicken embryo fibroblasts (CEFs), by means of hypotonic treatment and mechanical cell disruption. In this study, the structure of this subcellular fraction has been extensively characterised by using immunofluorescence analysis with antibodies against integrins and focal adhesion proteins, together with interference reflection microscopy and scanning force analysis. By using these techniques, we are able to demonstrate that VPM preparations maintain well-structured focal adhesions and stress fibers. Moreover the analysis on VPMs with antibodies specific for polypeptides postulated to act as crosslinkers between the membrane and the actin cytoskeleton, or as actin regulators, such as ezrin and Rac1 GTPase, has allowed the analysis of their distribution on the ventral side of the cell.

3.1.1 VPM preparations preserve focal adhesion sites

VPMs were prepared from adherent fibroblasts by a combination of hypotonic treatment and mechanical cell disruption, as described in chapter 2. These structures were visible by phase microscopy thanks to the presence of dense fibrils which largely overlapped with the distribution of actin filaments, as shown after staining with FITC-phalloidin (Fig.3.1A and 3.1B). Focal adhesions are visible along stress fibers and often at the end of them, when stained with anti-vinculin antibody (Fig. 3.1C). In Fig. 3.2,

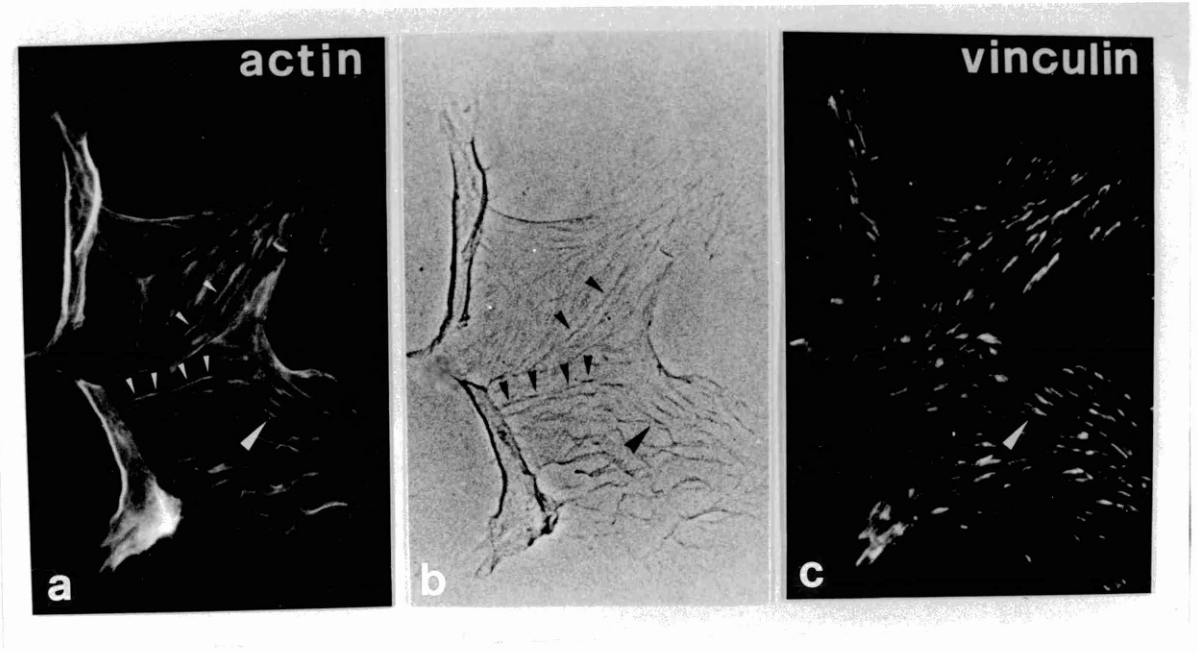


Figure 3.1. Phase contrast, actin and vinculin staining on VPM preparations.

VPMs were prepared, fixed and processed for immunofluorescence as described in chapter 2. The anti-vinculin monoclonal antibody was used to stain focal adhesions (Panel C). FITC-phalloidin was used to visualise actin stress fibers (Panel A), while a phase contrast view of the same VPM is visible in panel B. Arrowheads show colocalisation of structures on VPM. Bar = 10 μm.

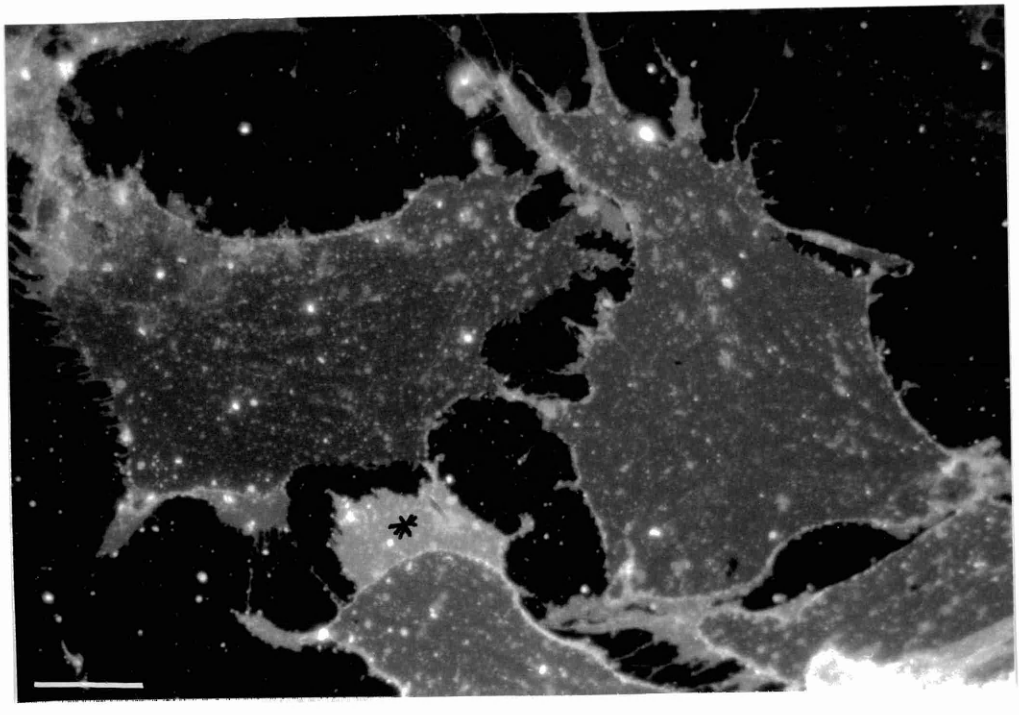


Figure 3.2. Phospholipid staining of VPMs.

After VPM preparations, membranes were stained with the lipophilic carbocyanine dye DiIC16 as described in chapter 2. The asterisk shows an area at the periphery of VPM were a portion of the dorsal plasma membrane is still present. Bar = 20 μm.

VPM preparations were stained with the fluorescent lipophilic dye DiIC16, in order to control that the phospholipid bilayer was not significantly damaged after the hypotonic and mechanical cell disruption.

By using an Interference Reflection Microscope (IRM), focal adhesions are visualised in intact cells as dark dots on a light surface, thanks to the fact that the part of the membrane engaged in focal adhesions is closer to the substrate (Block and Geiger, 1980). In order to investigate whether this property of the membrane was mantained in our preparations, we looked at VPMs with an IRM. As shown in two examples in Fig.3.3, dark areas corresponding to clusters of β1 integrins were still clearly visible on VPM preparations.

3.1.2 VPM preparations allow a clearer look at antigens on the ventral side of the cell

The distribution of the integrin β1 subunit and of vinculin appeared much clearer in VPM preparations as compared to intact cells (Fig. 3.4), due to the elimination of the fluorescent signal from the dorsal cell membrane, and from the cytoplasm. For the same reason, this system has revealed itself to be very useful for the study of the distribution on the membrane of other antigens that have a complex staining in intact cells. The small GTP-binding protein Rac1 showed a typical late endosomal, lysosomal pattern in intact cells, while it distributes along actin fibers and in focal adhesions in VPM preparations, as the costaining with vinculin indicates (Fig. 3.5).

Ezrin, radixin, and moesin (the ERM proteins) are closely related proteins known to function as membrane/cytoskeleton linkers (Tsukita and Yonemura, 1997; Tsukita *et al.*, 1997). The ERM proteins are found in dynamic plasma membranes/actin interfaces such as membrane ruffles, cleavage furrows, and microvilli; ezrin is a component of microvilli in a number of polarized epithelia (Bretscher, 1983; Hanzel et al., 1991; Berryman et al., 1993;, Franck et al., 1993;, Winckler et al., 1994; Amieva and Furthmayr, 1995). By using VPM preparations from adherent CEFs, we were able to demonstrate the presence of ezrin together with vinculin in focal adhesion sites, as shown in Fig. 3. 6.

3.1.3 Scanning force topography of VPMs

In order to obtain additional information about the structure of VPMs and of focal contacts, scanning force microscopy was performed on VPM preparations in parallel with immunofluorescence microscopy. A typical scanning force micrograph is a colour-coded

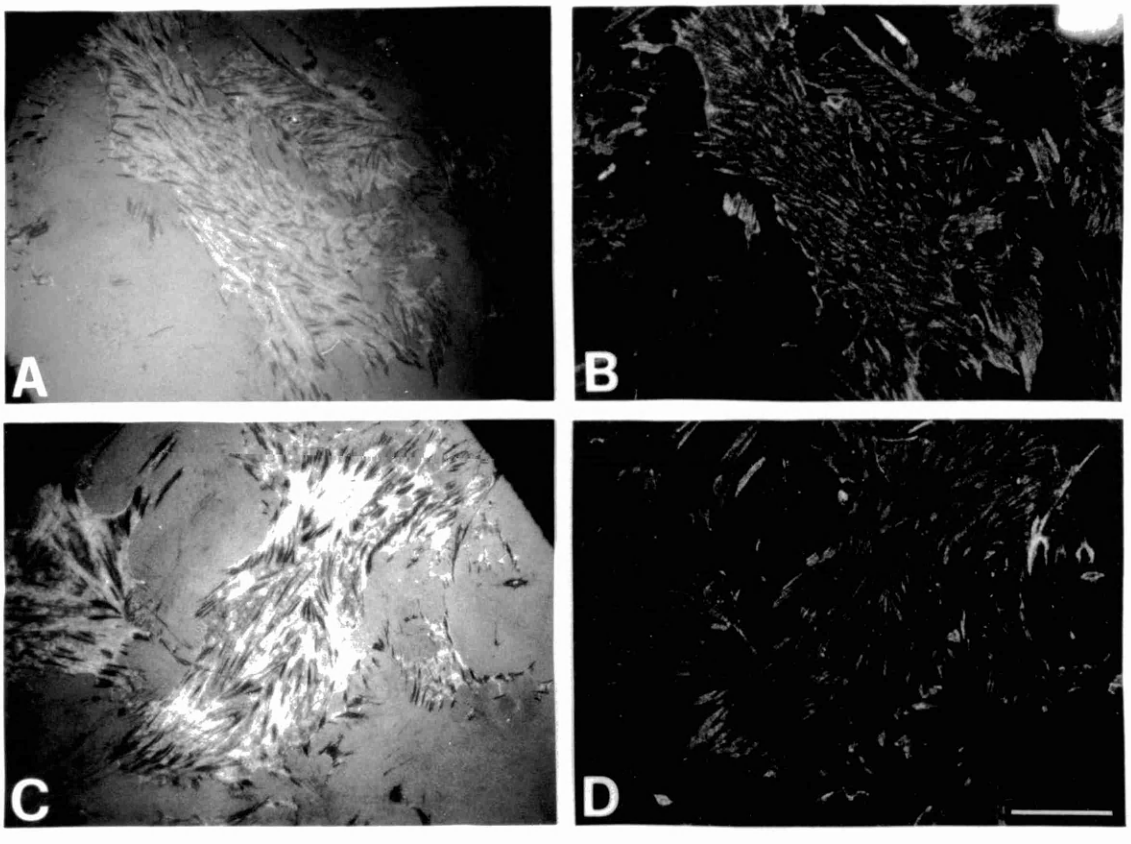


Figure 3.3. IRM and β1 integrins staining on VPMs.

VPMs were prepared and processed for immunofluorescence with β1-cyto polyclonal antibody against β1 integrins, as described in chapter 2. Panels B and D show b1 integrins staining in focal adhesions and diffused on the membrane, while in panels A and C the corresponding VPMs were observed with an Interference Reflection Microscope (IRM). Bar = 20 μm.

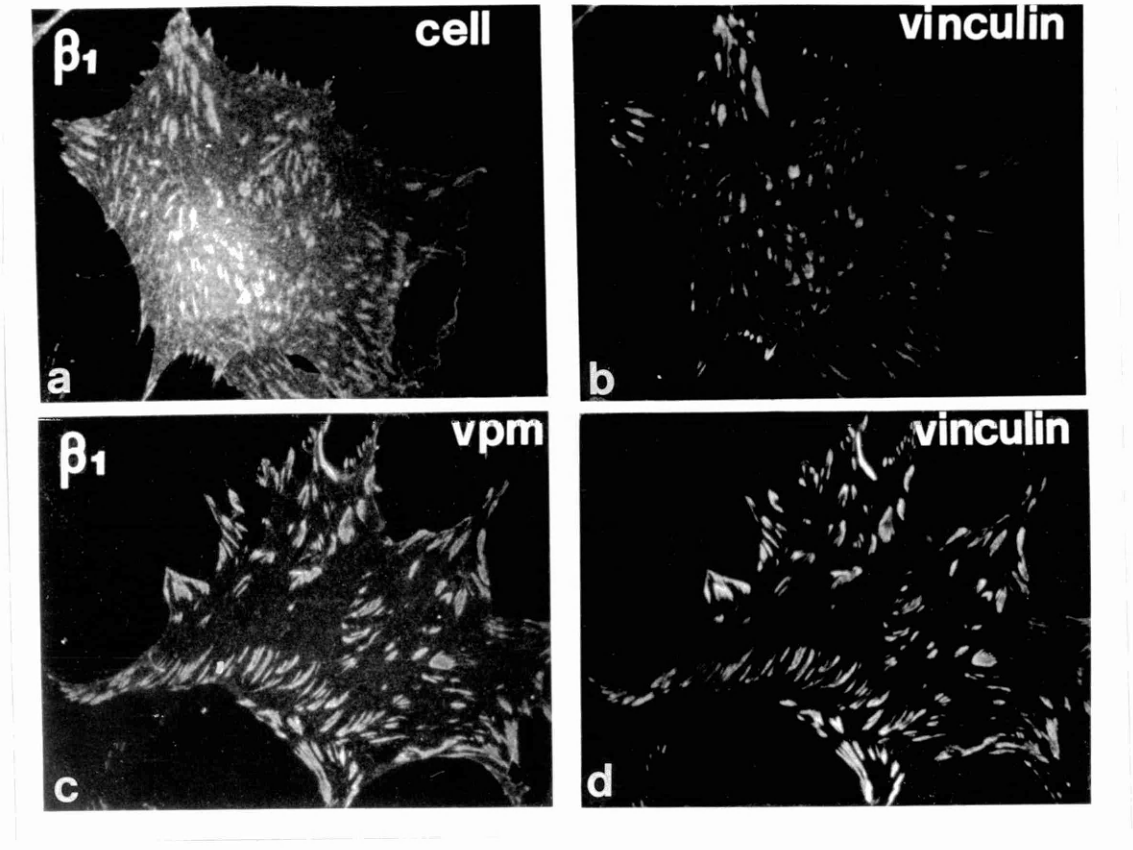


Figure 3.4. Distribution of the β1 integrin subunit and vinculin in intact cells and VPMs.

CEFs (panels A and B) and VPMs (panels C and D) were processed for immunofluorescence with the β1-cyto polyclonal antibody (panels A and C) and with the anti-vinculin monoclonal antibody (panels B and D). The same cell is shown in panels A and B, and the same VPM in panels C and D. Bar = 10 μm.

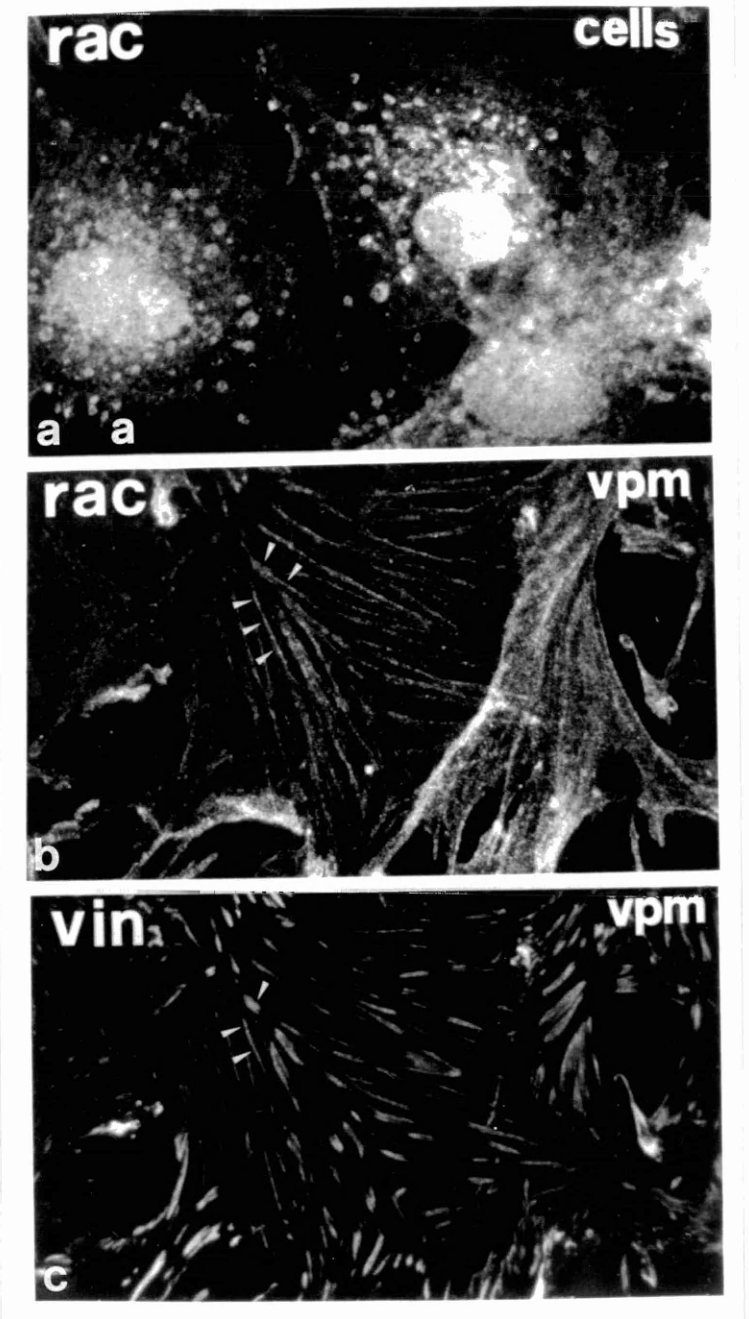


Figure 3.5. Rac 1 distribution on VPMs.

CEFs (panel A) and VPMs (panels B and C) were stained with the anti-Rac 1 polyclonal antibody (panels A and B) and with the anti-vinculin monoclonal antibody (panel C). The same membrane is shown in panel B and C. Arrowheads in panels B and C indicate areas of colocalisation of Rac 1 and vinculin. Bar = 10 μm.

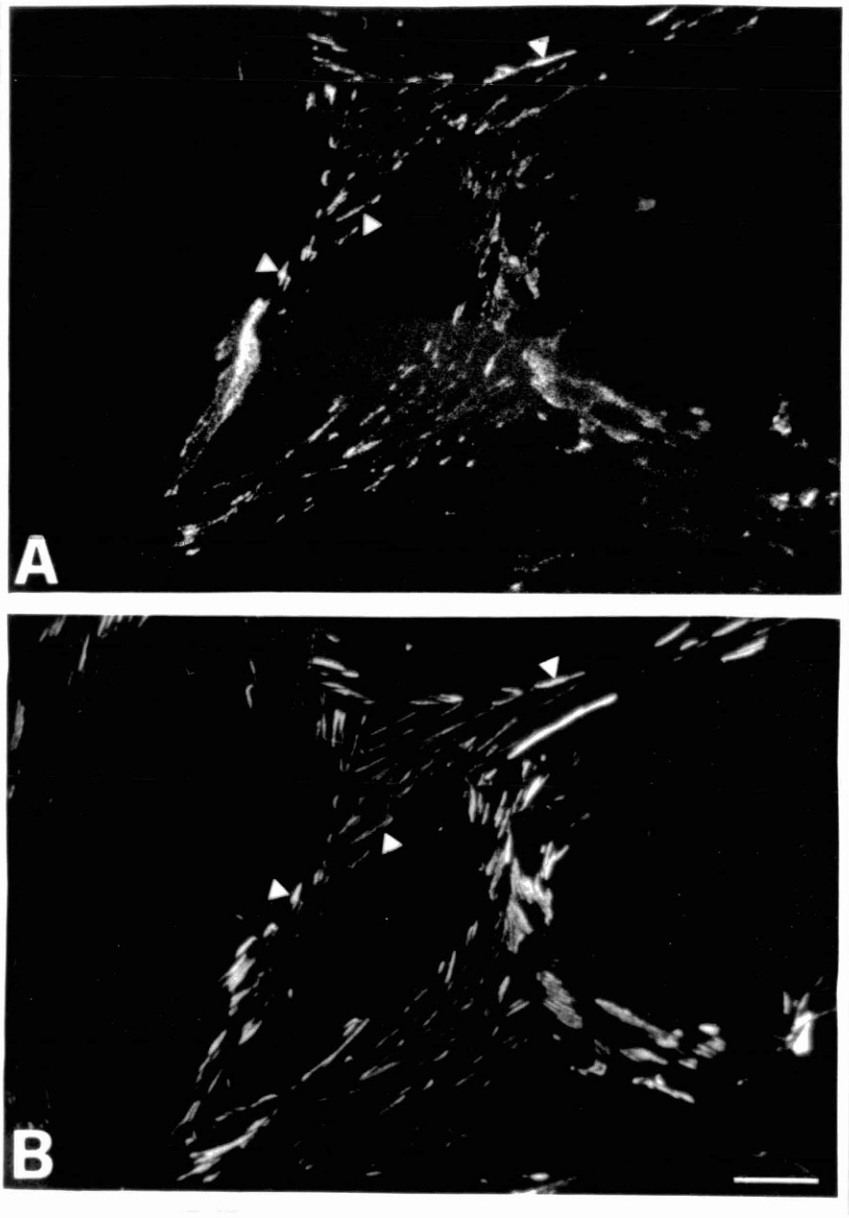


Figure 3.6. Ezrin colocalises with vinculin in focal adhesions on VPMs.

VPMs were processed for immunofluorescence using the anti-ezrin polyclonal antibody (panel A) and the anti-vinculin monoclonal antibody (panel B). The same membrane is shown in the two panels and arrowheads indicate sites of colocalisation between the two proteins. Bar = 10 μm.

topograph obtained by a digital recording of the separation necessary to maintain a constant force between a spring-mounted stylus and a sample surface throughout a serial, two-dimensional scan of the surface.

One of the most striking aspects of the topographs of VPMs was the complete view of the complex weave of components that resulted. Fig. 3.7 is an example of the thorough view of VPM structure provided by scanning force microscopy. In Fig. 3.7A, a VPM lay beside an unsheared cell (arrow). The height of the intact cell exceeded the vertical scan range of the microscope and thus saturated the colour-coded height scale. Fine details of the surface of the intact cell were not visible, while the nearby VPM had clearly distinguished stress fibers that radiated from the centre. The terminations of the stress fibers were clearly depicted in a higher magnification image, Fig. 3.7B. In addition to high contrast detection of focal adhesion sites and stress fibers, small holes in the membrane (Fig. 3.7A, arrowheads) and small particles of approximately 100 nm-diameter (Fig. 3.7B, arrowheads) were observed. These features were not detected in immunofluorescence or phase contrast micrographs of the membrane (see Fig. 3.1).

In another example, a VPM exhibited a pair of prominent actin bundles (Figs 3.8C and 3.8D, arrows) which appeared to connect directly to focal contacts (Figs 3.8B and 3.8D, arrowheads). Actin in the fibers and vinculin in the focal contacts was identified using immunofluorescence microscopy, but the precise alignment at these junctions was succinctly displayed in the topograph (Fig. 3.8D). A damaged area of this ventral plasma membrane was visible in scanning force micrographs (Fig. 3.8A, dark outline).

Unidentified filaments were also detectable under and around the ventral plasma membranes. In both Fig. 3.9A (15 hours culture) and Fig. 3.9C (3 hours culture) the perimeters of the membranes were obvious and many fibers were found crossing the membranes. Fluorescent, actin labelling was delimited by the membrane perimeters and numerous parallel stress fibers were visible on the membrane of a cell cultured overnight (Fig. 3.9B) or for 3 hours (Fig. 3.9D). On both membranes there were also filaments that lay transverse to the predominant orientation (arrows in Figs 3.9A, 3.9C, 3.9E, and 3.9F). In the fluorescence image (Fig. 3.9B), actin labelling did not reveal the transverse filaments crossing the membrane of Fig. 3.9A (also Fig. 3.9F), while the transverse fibers in Fig. 3.9C (also Fig. 3.9E) were clearly labelled in Fig. 3.9D. The unlabelled, transverse filaments were continuous with filaments that extended beyond the perimeter of the membrane (Fig. 3.9A), but they did not colocalise with anti-vinculin labelling (data

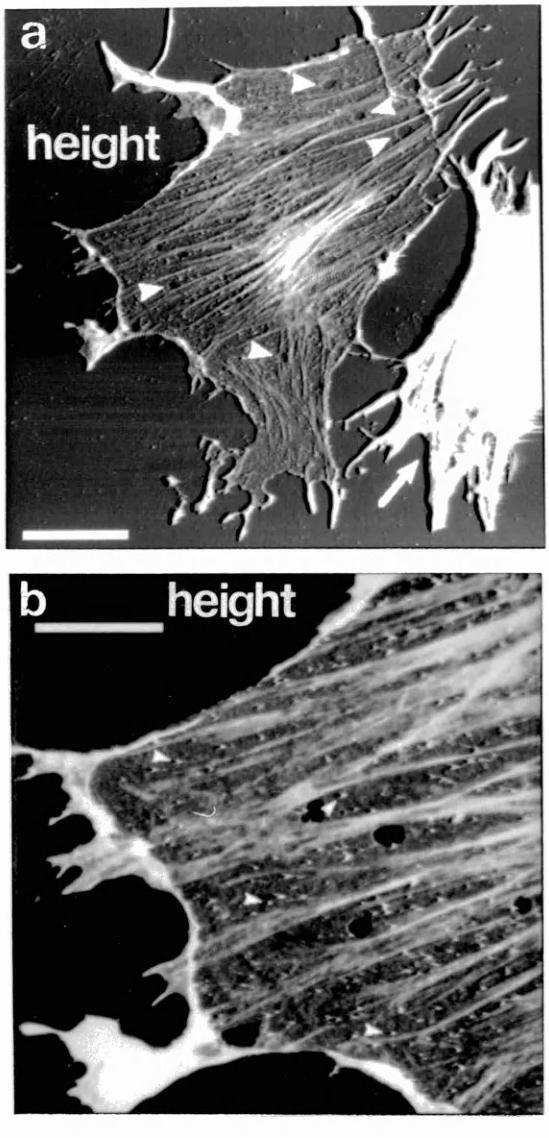


Figure 3.7. Scanning force micrograph of a VPM.

Panel A: a scanning force micrograph of a VPM from a cell cultured 3 hours on fibronectin-coated glass. Small holes in the membrane (arrowheads) were visible as well as an intact cell (arrow). Panel B: a higher magnification, scanning force micrograph of the left-hand edge of the same ventral plasma membrane revealed numerous small particles (arrowheads). Bar = 10 μm in panel A and 4 μm in panel B. Colour-coded height range = 800 nm in panel A and 200 nm in panel B.

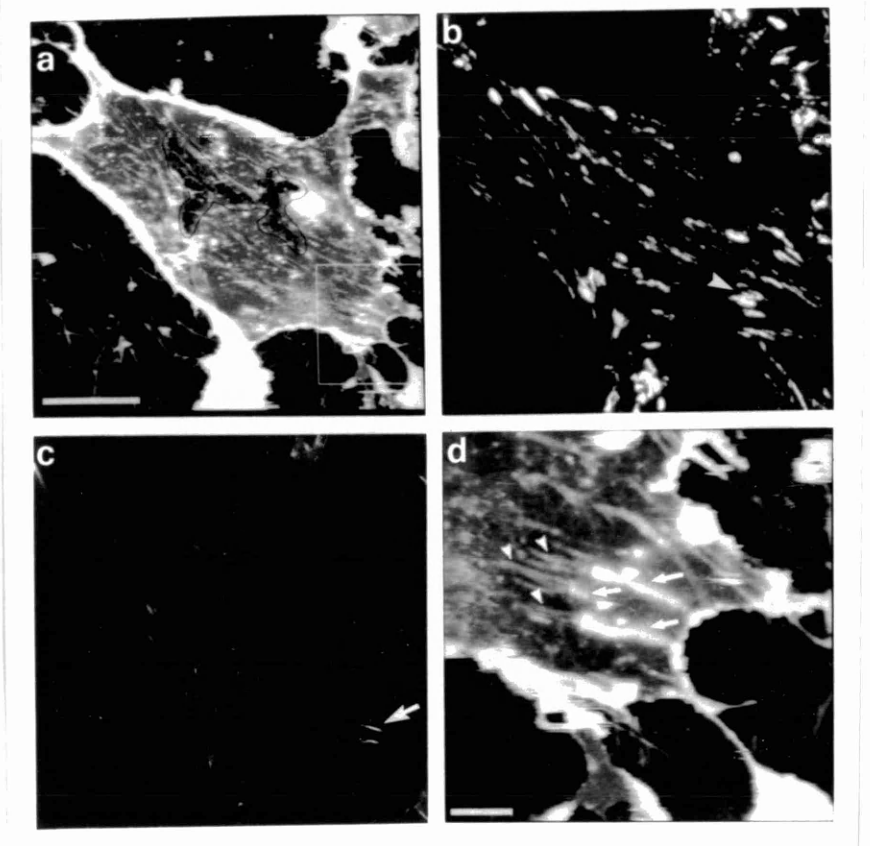


Figure 3.8. Scanning force, vinculin and actin staining of a VPM.

Panel A: a scanning force micrograph of a ventral plasma membrane from a cell cultured 15 hours on glass. Some damage to the membrane (dark outline) was evident. The same membrane was labelled by immunofluorescence with the anti-vinculin monoclonal antibody (panel B), or with phalloidin for F-actin staining. Vinculin-labelled focal adhesions (arrowheads) and actin-labelled stress fibers (arrows) are indicated. Panel D: a higher magnification scanning force micrograph of the boxed area in panel A, showing the focal adhesions (arrowheads) and stress fibers (arrows) indicated in panels B and C. Scale bars: panels A, B and C = 10 μm, panel D = 2 μm. Colour-coded height ranges: panel A = 450 nm, panel D = 350 nm.

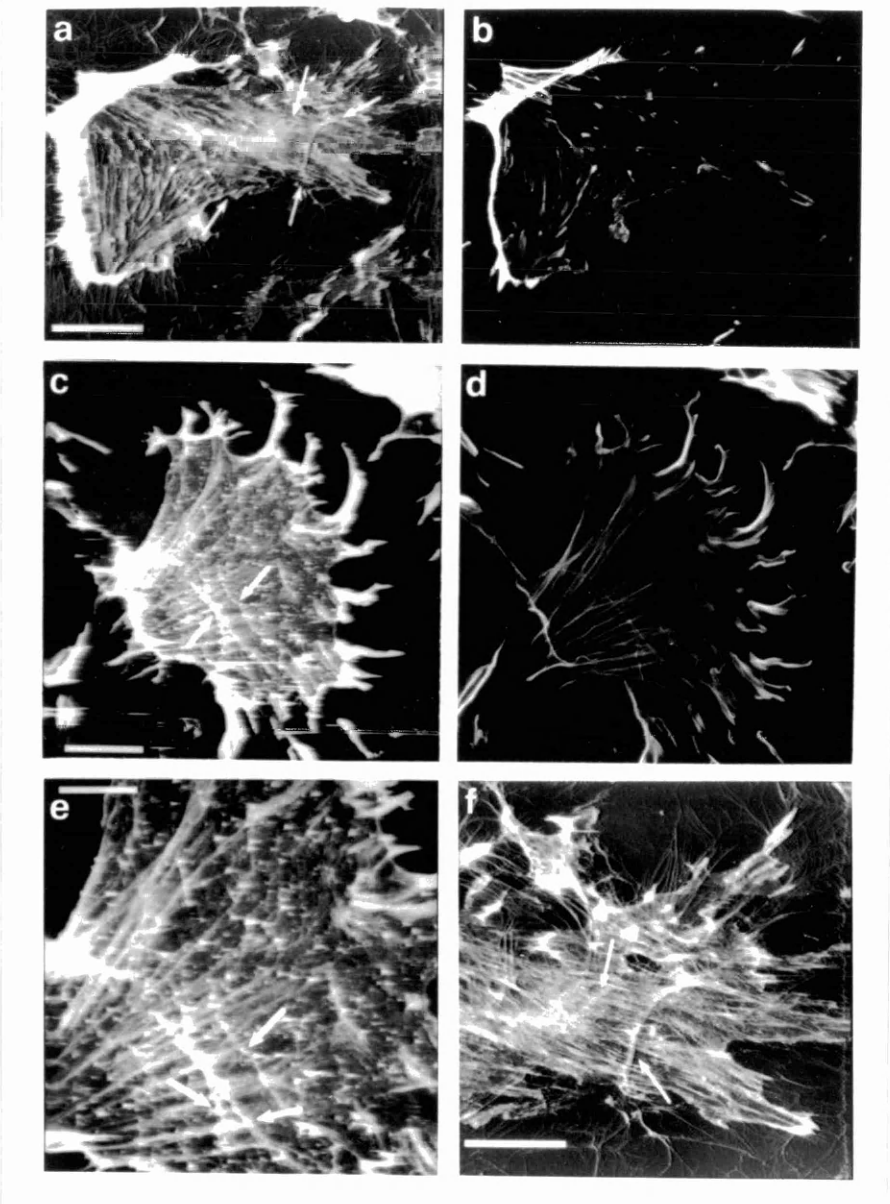


Figure 3.9. Scanning force micrograph and actin staining of VPMs prepared from CEFs cultured for different times.

Panel A: a scanning force micrograph of a ventral plasma membrane from a cell cultured 15 hours on glass and (panel B) immunofluorescent, F-actin labelling of the same ventral plasma membrane. Arrows indicate filaments oriented transversely with respect to the majority of filaments. Panel C: a scanning force micrograph of a ventral plasma membrane from a cell cultured 3 hours on fibronectin-coated glass and (panel D) immunofluorescent, F-actin labelling of the same ventral plasma membrane. Panels E and F: higher magnification scanning force micrographs of the transverse filaments indicated in panel C and A (respectively). Scale bars: panels A, B, C and D = 20 μm, panel E 5 = μm, panel F = 10 μm. Color-coded height ranges: panel A = 500 nm, panels C and E = 250 nm, panel F = 400 nm.

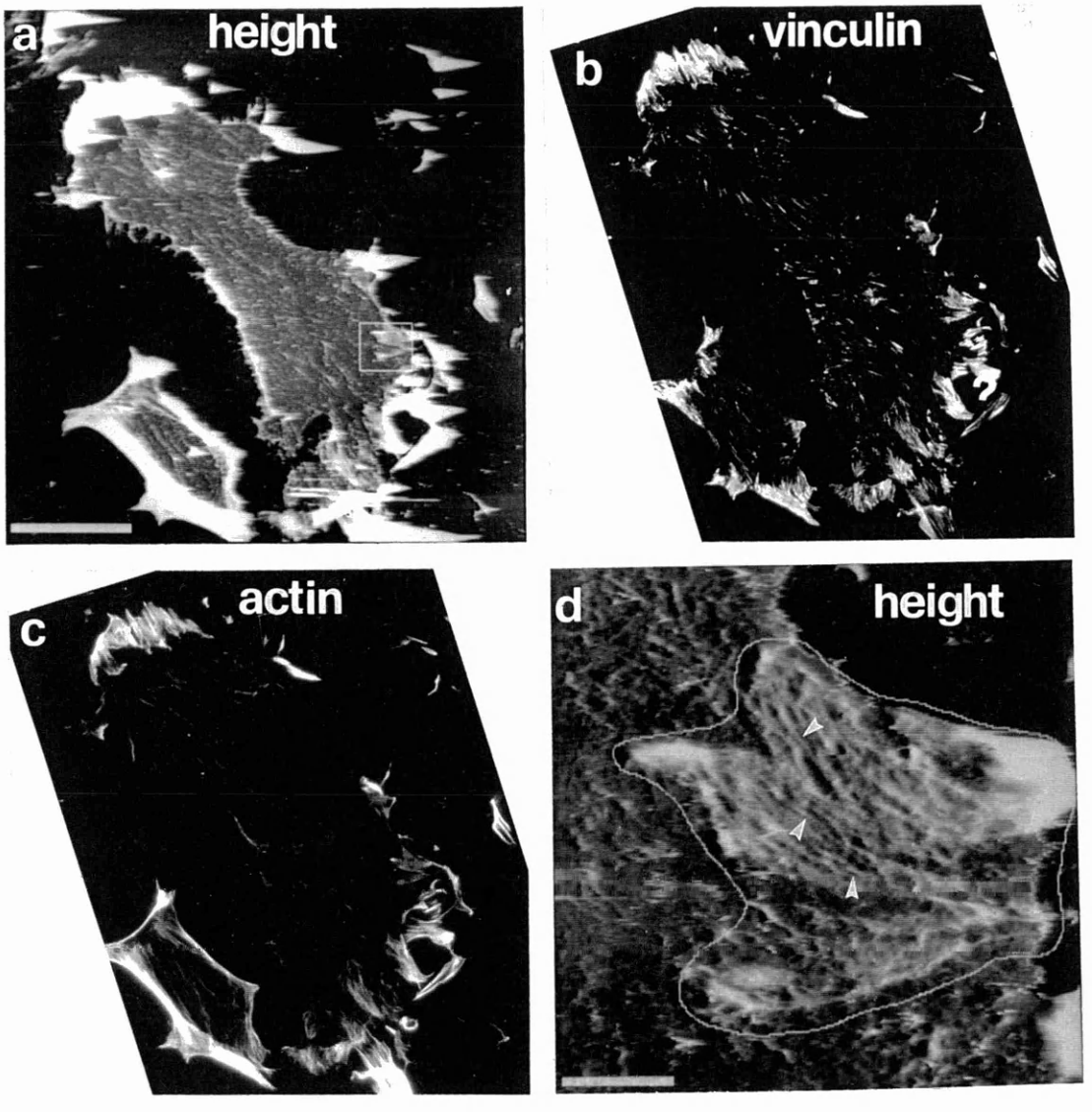


Figure 3.10. Scanning force micrograph and immunofluorescent labelling of a VPM from short term culture.

Panel A: a scanning force micrograph of a ventral plasma membrane from a cell cultured 3 hours on fibronectin-coated glass. Immunofluorescent labelling of vinculin (panel B) and actin (panel C) on the same ventral plasma membrane. Panel D: a higher magnification scanning force micrograph of the focal contact in the boxed area in panel A. Arrows indicate 60 nm actin-containing fibrils. Scale bars: panels A, B and C = 20 μm, panel D = 2 μm. Color-coded height ranges: panels A and D = 200 nm.

not shown), as reported previously for cable-like extracellular matrix structures lying under ventral plasma membranes (Chen and Singer, 1982).

The areas surrounding VPMs differed greatly according to the duration of the cell culture. After an overnight culture (Fig. 3.9A) the substrate was strewn with filaments that were not labelled by fluorescent phalloidin (Fig. 3.9B) and therefore did not appear to be actin. Instead for ventral plasma membranes prepared after brief cultures (Fig. 3.9C) the surrounding substrate was free of such filaments.

When fibroblasts were cultured for 1.5-3 hours, they formed large focal adhesions on their peripheries, as we have previously published (Cattelino *et al.*, 1995). VPMs prepared from these brief cultures were often free of overlying stress fibers. The VPM shown in Fig. 3.10A had broad focal contacts revealed by fluorescent labelling of vinculin in Fig. 3.10B. In a high magnification scanning force micrograph of the focal contact (Fig. 3.10D), fine parallel fibers as small as 60 nm in width were revealed (arrowheads). These fibers were probably very thin bundles of actin filaments associated with vinculin and other focal contact proteins, since this focal contact was also lightly stained for actin (Fig. 3.10D). Such fine fibers were not resolved in the fluorescence micrograph at 1000 X.

3.2 PREFERENTIAL LOCALISATION OF TYROSINE-PHOSPHORYLATED PAXILLIN IN FOCAL ADHESIONS

Focal adhesions are formed by large clusters of integrins and by a large number of intracellular proteins which accumulate at these sites, and that seem to be important both for signalling and cytoskeletal reorganisation. The mechanism of assembly of focal adhesions and the role of the numerous proteins colocalising with these adhesive structures is still poorly understood. Tyrosine phosphorylation is known to be an early event in integrin-mediated signalling (Schaller and Parsons, 1993), and integrin clustering is an important event for the recruitment of several cytoplasmic proteins at the adhesive sites (Miyamoto et al., 1995). Paxillin and focal adhesion kinase (FAK), a non-receptor tyrosine kinase, are among the best characterised proteins colocalising with integrins in focal adhesions (Turner *et al.*, 1990; Schaller *et al.*, 1992; Schaller and Parsons, 1993; Turner, 1994). FAK and paxillin are highly phosphorylated on tyrosine residues during early embryonic development (Turner, 1991; Turner *et al.*, 1993), upon

integrin-mediated cell adhesion or antibody-induced integrin clustering (Kornberg *et al.*, 1991; Burridge *et al.*, 1992), and in response to a variety of mitogens (Zachary *et al.*, 1992; Zachary *et al.*, 1993; Rankin and Rozengurt, 1994). The finding that paxillin tyrosine-phosphorylation is accompanied by a similar increase in the tyrosine phosphorylation of FAK (Burridge *et al.*, 1992) suggests that the activity of FAK and paxillin tyrosine phosphorylation may be closely related, although their exact role in focal adhesion assembly and cytoskeleton reorganisation is not known yet. Furthermore, tyrosine-phosphorylated FAK and paxillin polypeptides usually represent a minor fraction of the total polypeptide in non-transformed cells, and the role of tyrosine phosphorylation of these proteins *in vivo* remains to be established.

The utilisation of VPM preparations for biochemical and morphological characterisation is described in this part, by using antibodies raised against different components of the focal adhesions. The results from this study show the accumulation of several tyrosine-phosphorylated proteins in VPMs, including tyrosine-phosphorylated FAK and paxillin. In particular, our findings identify a pool of highly tyrosine-phosphorylated paxillin accumulating in focal adhesions, suggesting an important role of the phosphorylated polypeptide in the mechanisms of integrin-mediated adhesion.

3.2.1 VPMs enriched in β1 integrins contain focal adhesion components and are enriched in tyrosine-phosphorylated polypeptides

We have used a preparation of VPMs to biochemically characterise the adhesion sites formed by fibroblasts on extracellular matrix. Intact cells and VPM preparations were extracted with 1% Triton X-100 lysis buffer, and equal amounts of protein from the solubilised fractions were analysed by immunoblotting with different primary antibodies. VPM extracts were highly enriched in the integrin β1 polypeptide, a major transmembrane component of focal adhesions (Fig. 11A). VPMs showed a less dramatic enrichment for non-membraneous, cytosolic components of the focal adhesions, such as talin and vinculin, although immunofluorescence showed a clear colocalisation of these proteins with β1 integrins at the adhesion sites found in VPMs prepared from CEFs (see Fig.3.4 for vinculin). These results suggest that only a fraction of these polypeptides could be recovered in a membrane-associated form in these cells. Moreover the results show that most of these proteins were solubilised by Triton X-100 from VPMs, while a fraction of actin was recovered in the Triton-insoluble pellet (Fig. 3.11A, lane 3).

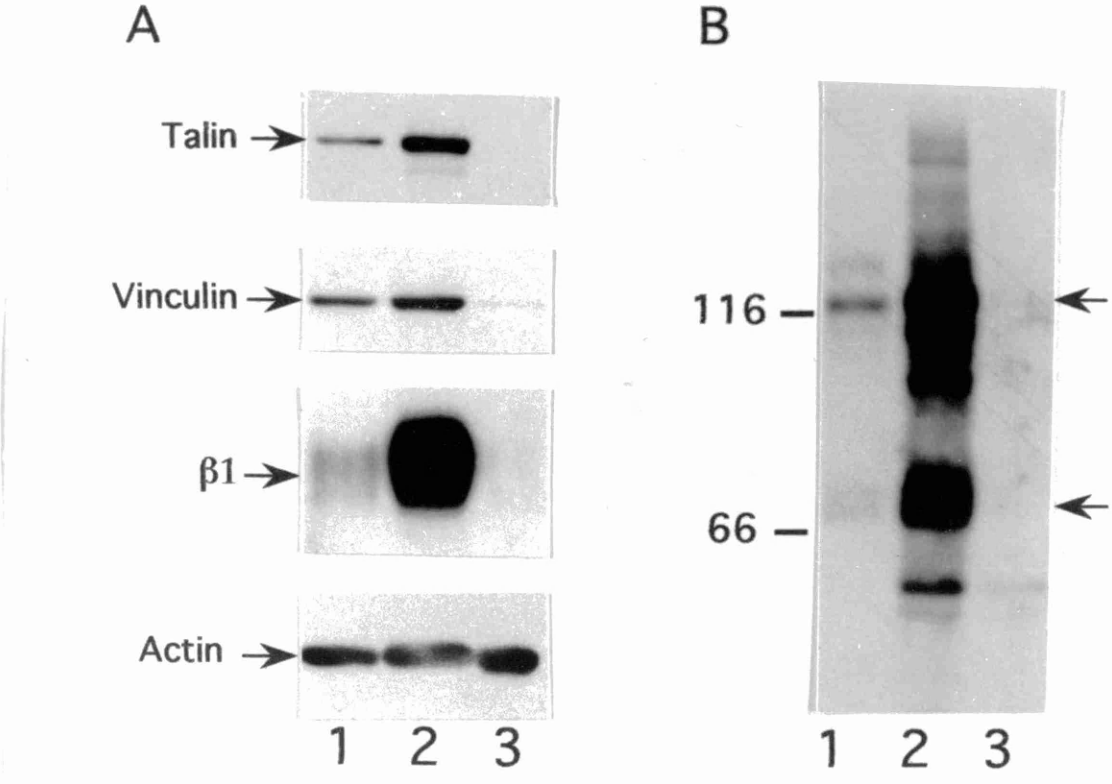


Figure 3.11. Accumulation of focal adhesion components and tyrosine-phosphorylated polypeptides in VPMs.

CEFs were cultured for 18 h, and VPMs were prepared as described in chapter 2. Equal amounts of protein from Triton X-100 extracts of intact CEFs (lanes 1) and of VPMs (lanes 2), and from the Triton X-100-insoluble fraction of VPMs (lanes 3) were analysed by immunoblotting with antibodies against the indicated proteins (panel A), or against phosphotyrosine (panel B). In panel B, molecular mass markers are indicated to the left of the blot; the arrows point to two major tyrosine-phosphorylated polypeptides of 120 kDa and 68 kDa.

The analysis of tyrosine-phosphorylated polypeptides showed a clear increase in the signal of a number of bands in VPM preparations when equal amounts of whole cell extracts and of extracts from VPMs were compared (Fig. 3.11B). Immunofluorescence analysis of intact, fixed and permeabilised CEFs with anti-phosphotyrosine antibodies showed a clear localisation of phosphotyrosine at focal adhesion sites, together with a more diffuse, dotted intracellular staining (Fig. 3.12A). On the other hand, anti-phosphotyrosine antibodies stained only focal adhesions in VPMs, indicating that the observed enrichment of tyrosine-phosphorylated proteins in the VPM preparations is due to accumulation in focal adhesions (Fig. 3.12B).

3.2.2 Accumulation of Tyrosine-Phosphorylated FAK in VPMs

A major tyrosine-phosphorylated band of approximately 120 kDa was visible by immunoblotting with anti-phosphotyrosine antibodies both in whole cell and VPM lysates (Fig. 3.13). Interestingly, a small, but reproducible difference could be observed in the mobility of the major tyrosine-phosphorylated band of about 120 kDa between CEFs and VPMs (Fig. 3.13, lanes 1 and 2, respectively). To check whether this band corresponded to tyrosine phosphorylated FAK, we immunoprecipitated FAK from equal amounts of protein from CEF and VPM lysates, and analysed the immunoprecipitates by immunoblotting with anti-phosphotyrosine antibodies (Fig. 3.13, lanes 3 and 4). Anti-FAK antibodies specifically immunoprecipitated the 120 kDa tyrosine-phosphorylated band, which was enriched in VPMs compared to CEFs. Although tyrosine-phosphorylated FAK was enriched in VPM compared to whole cell lysates, the VPM lysate could not be completely depleted of the 120 kDa tyrosine-phosphorylated band by immunoprecipitation with anti-FAK antibodies (Fig. 3.13, lane 6). Longer exposures of the autoradiogram show that depletion of the 120 kDa polypeptide was incomplete also in the whole cell lysate (not shown). Since we were able to deplete the lysates obtained from other chick cell types of tyrosine-phosphorylated FAK by using the same antibody (not shown), we think that the presence of a tyrosine-phosphorylated 120 kDa protein in the unbound fraction of CEFs and VPMs was not due to inefficient immunoprecipitation of the antigen, but was probably due to the presence of a different protein comigrating with FAK.

Analysis of the distribution of FAK by immunofluorescence showed that FAK could only be detected in focal adhesions in VPM preparations (Fig. 3.14B). In intact,

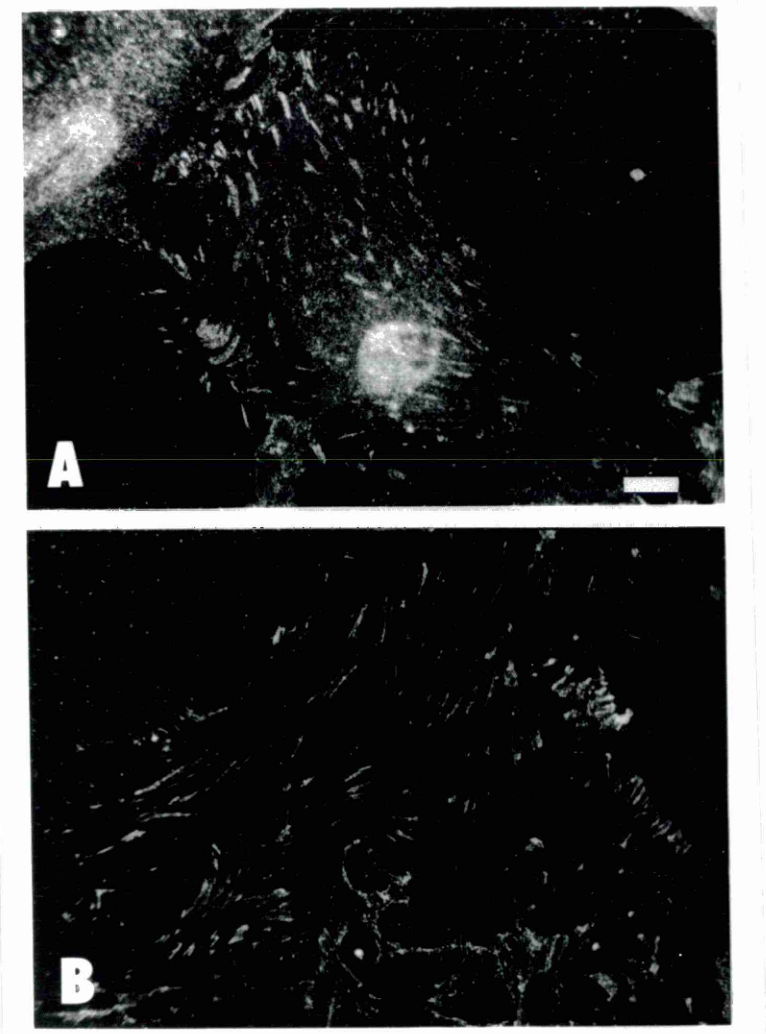


Figure 3.12. Distribution of tyrosine-phosphorylated polypeptides in focal adhesions.

Intact CEFs (panel A) and VPMs (panel B) were fixed and permeabilised as described in chapter 2. Incubation with anti-phosphotyrosine antibodies was followed by incubation with biotin-conjugated anti-mouse immunoglobulin and Texas red-conjugated streptavidin. Bar = 10 μm.

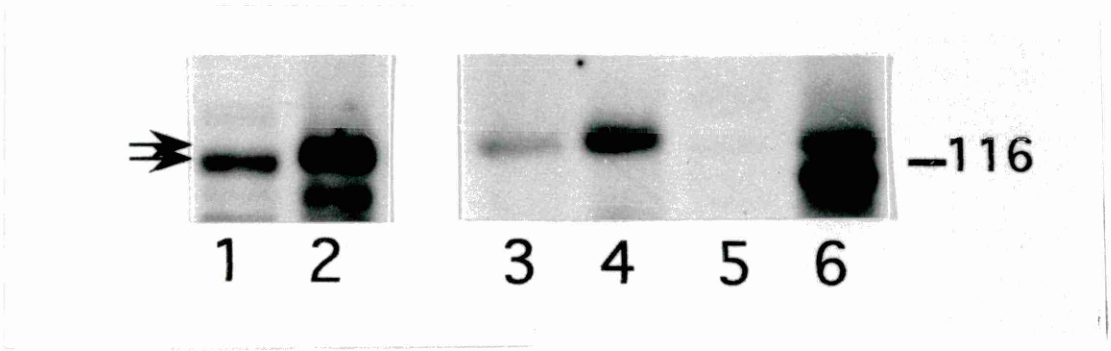


Figure 3.13. Enrichment of tyrosine-phosphorylated FAK in VPMs.

(pAb Tarone)

FAK was immunoprecipitated from equal amounts of protein from CEF (lane 3) and VPM (lane 4) lysates. Immunoprecipitates, equal amounts (40 μg) of lysates (lanes 1, CEFs; lane 2, VPMs), and about half of each of the non-bound fractions from the immunoprecipitates (lanes 5 and 6, respectively) were blotted and incubated with anti-phosphotyrosine antibodies. The two arrows to the left of the blot point to the two tyrosine-phosphorylated bands of about 120kDa visible in the CEF and VPM lysates, respectively. The 116 kDa molecular mass marker is indicated to the right.

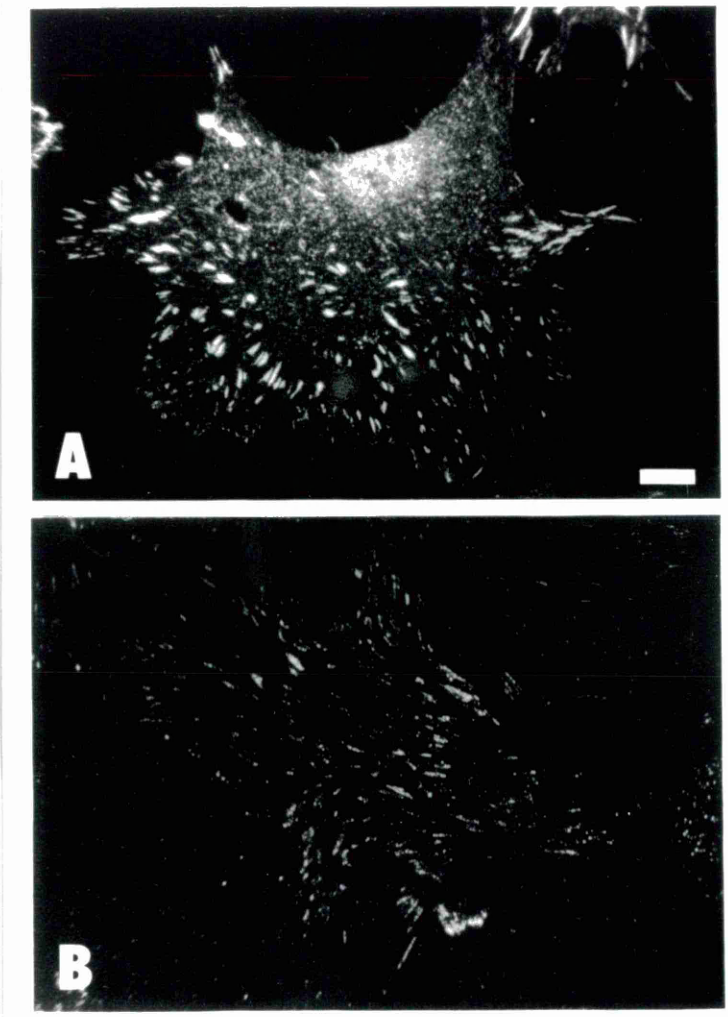


Figure 3.14. Distribution of FAK to focal adhesions in VPMs.

Intact CEFs (panel A) and VPMs (panel B) were fixed and permeabilised as described in chapter 2. Incubation with anti-FAK antibodies was followed by incubation with biotin-conjugated anti-mouse immunoglobulin and Texas red-conjugated streptavidin. Bar = 10 μm.

permeabilised CEFs, FAK distribution was characterised by localisation to focal adhesions, and by a diffuse, cytoplasmic punctate staining (Fig. 3.14A).

3.2.3 Enrichment of Tyrosine-Phosphorylated Paxillin in VPMs

We analysed preparations of VPMs for the presence of paxillin. Analysis of equal amounts of protein from lysates from whole cells and from VPMs (Fig. 3.15A) showed that the relative concentration for paxillin was similar in the two preparations, with a 0.8-fold enrichment in VPMs compared to whole cell lysate. Interestingly, a reproducible shift was observed for the paxillin detected in VPMs, which migrated on the gels with a slightly slower mobility compared to paxillin from whole cell extracts (Fig. 3.15A, lanes 2 and 1, respectively). Analysis of equal amounts of protein from the same preparations by immunoblotting with anti-phosphotyrosine antibodies revealed a band of about 68 kDa comigrating with the VPM form of paxillin, which was strongly enriched in VPMs compared to whole cell extract (Fig. 3.15A, compare lanes 4 and 3, respectively). As shown in Figure 3.15B, immunoprecipitation with anti-paxillin antibodies was able to immunodeplete the tyrosine-phosphorylated 68 kDa polypeptide from VPM lysates, showing that this phosphorylated band corresponded to the tyrosine-phosphorylated paxillin. Stripping and re-blotting of the same filters with anti-paxillin antibodies confirmed that similar amounts of the paxillin polypeptide could be immunoprecipitated from equal amounts of protein from whole cell and VPM lysates (Fig. 3.15C).

The recovery of several focal adhesion components in VPMs was quantified from blots by densitometry as described in the Methods. Quantitation showed that only 2.6% (±1.4, s.e.; n=2) of the total Triton-X-100 extractable protein was recovered in VPM lysates, and that more than 33% (±13.6, s.e.; n=2) of the transmembrane integrin β1 subunit present in the cells was recovered in VPM lysates. Only a minor fraction of cytosolic focal adhesion components, such as talin, vinculin and paxillin, were recovered in VPM preparations (not shown). On the contrary, 36% (±12.7, s.e.; n=2) of tyrosine-phosphorylated paxillin was recovered in VPM lysates, indicating a specific accumulation of this phosphorylated polypeptide in VPMs. In Figure 3.16, the enrichment of focal adhesion components in VPM lysates compared to total CEF lysates is shown. Enrichments were calculated as described in chapter 2. No enrichment was detectable for paxillin in VPMs (0.7-fold), while a strong enrichment (14.2-fold) in the tyrosine-phosphorylated form of paxillin could be observed in the same preparations. A similar enrichment (12.6-fold) was calculated for the integrin β1 subunit.

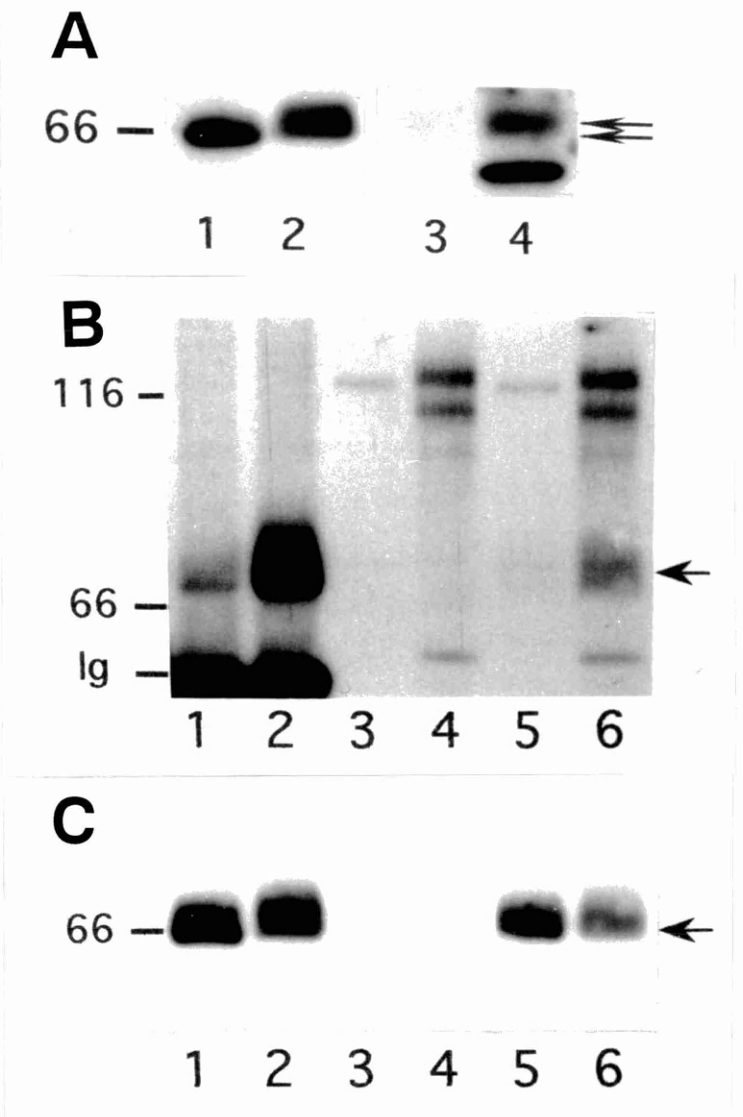


Figure 3.15. A tyrosine-phosphorylated 68 kDa band highly enriched in VPMs.

Panel A: CEFs (lanes 1 and 3) and VPMs (lanes 2 and 4) prepared from CEFs cultured on fibronectin-coated dishes were lysed as described in chapter 2. Lysates were fractionated by SDS-PAGE, transferred to nitro-cellulose, and incubated with anti-paxillin (lanes 1 and 2), or with anti-phosphotyrosine (lanes 3 and 4) antibodies. The 66 kDa molecular mass marker is indicated to the left. The two arrows to the right indicate the difference in migration of paxillin in lanes 1 and 2. Panel B: paxillin was immunoprecipitated from 130 μg of protein from CEF or VPM lysates (lanes 1 and 2, respectively). Immunoprecipitates, and same amounts of non-bound fractions (lanes 3 and 4, respectively) and of lysates (lanes 5 and 6, respectively) were blotted and incubated with anti-phosphotyrosine antibodies. Panel C: the same blot shown in panel B was stripped as described in chapter 2, and reblotted with the anti-paxillin antibody. Molecular mass markers are indicated to the left of the blots. Paxillin (panel C), and tyrosine-phosphorylated paxillin (panels A and B) are indicated by the arrows to the right. No evident differences in paxillin phosphorylation were observed when cells were plated on fibronectin (panel A) or directly on plastic (panel B).

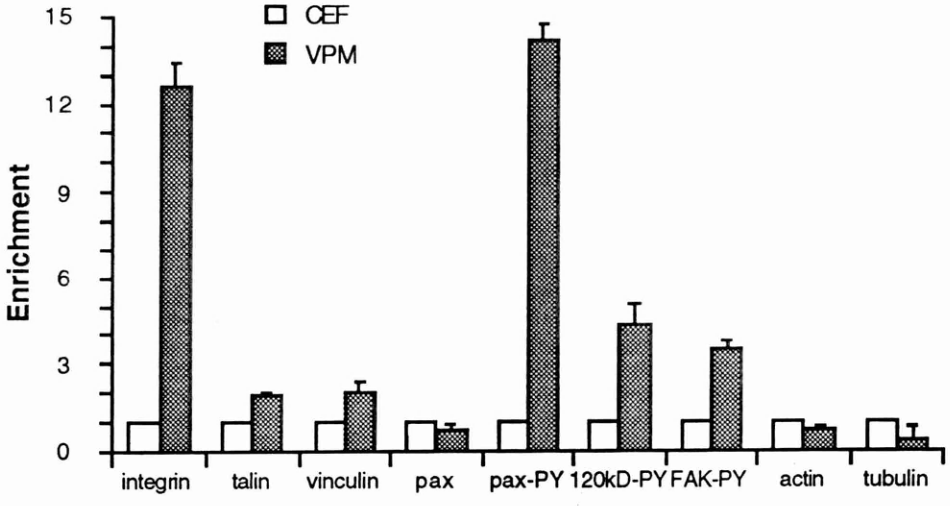


Figure 3.16. Enrichment of focal adhesion components in VPMs.

Lysates from CEFs and VPMs were analysed for protein content, and by immunoblotting for the presence of focal adhesion components, as shown in Figures 11, 13 and 15. Enrichments of polypeptides in VPMs were calculated as the ratio between the relative concentrations in VPM and in CEF lysates, as detailed in chapter 2. The values are expressed as the average from two experiments. Bars = standard deviation.

Localisation by immunofluorescence in CEFs showed that anti-paxillin antibodies gave both a diffuse, cytoplasmic staining, and a focal adhesion pattern (Fig. 3.17A). Staining of VPMs was more defined, and concentrated uniquely in focal adhesions, showing loss of the diffuse staining upon cell disruption (Fig. 3.17C).

3.2.4 Comparison of the level of Tyrosine-Phosphorylation of paxillin in CEFs and in VPMs

The different percentages of total paxillin and of tyrosine-phosphorylated paxillin recovered in VPM preparations indicated that the levels of tyrosine phosphorylation of paxillin in CEF and VPM lysates were different. To quantitatively analyse the differences in the level of tyrosine phosphorylation between the populations of paxillin recovered from whole cell lysates versus VPM lysates, we immunoprecipitated total paxillin and tyrosine-phosphorylated paxillin from equal amounts of protein from CEF lysates, by using anti-paxillin, and anti-phosphotyrosine antibodies, respectively. Immunoprecipitates and non-bound fractions after immunoprecipitation were analysed by immunoblotting with anti-phosphotyrosine (Fig. 3.18A), and anti-paxillin antibodies (Fig. 3.18B). Both antibodies were able to immunodeplete the lysates from tyrosine-phosphorylated paxillin (Fig. 3.18A, lanes 3 and 4). On the other hand, only the anti-paxillin antibody was able to immunodeplete the lysate of paxillin (Fig. 3.18B, lane 4). The anti-phosphotyrosine antibody was able to immunoprecipitate only the tyrosine-phosphorylated, 68 kDa form of paxillin (Fig. 3.18B, lane 1, upper arrow), while most of paxillin, corresponding to the non-tyrosine-phosphorylated polypeptide, was recovered in the non-bound fraction (Fig. 3.18B, lane 3).

The bands from the blot shown in Figure 3.18 were quantitated by densitometry, and the ratio between the values from lanes 1A and 1B was considered equivalent to a level of 100% tyrosine phosphorylation of paxillin (Fig. 3.19, PY-CEF). By using this as a reference, we found that only about 5% of total paxillin was tyrosine-phosphorylated in whole cell lysates (Fig. 3.19, PAX-CEF), while the level of paxillin tyrosine phosphorylation in VPMs was 90% (Fig. 3.19, PAX-VPM).

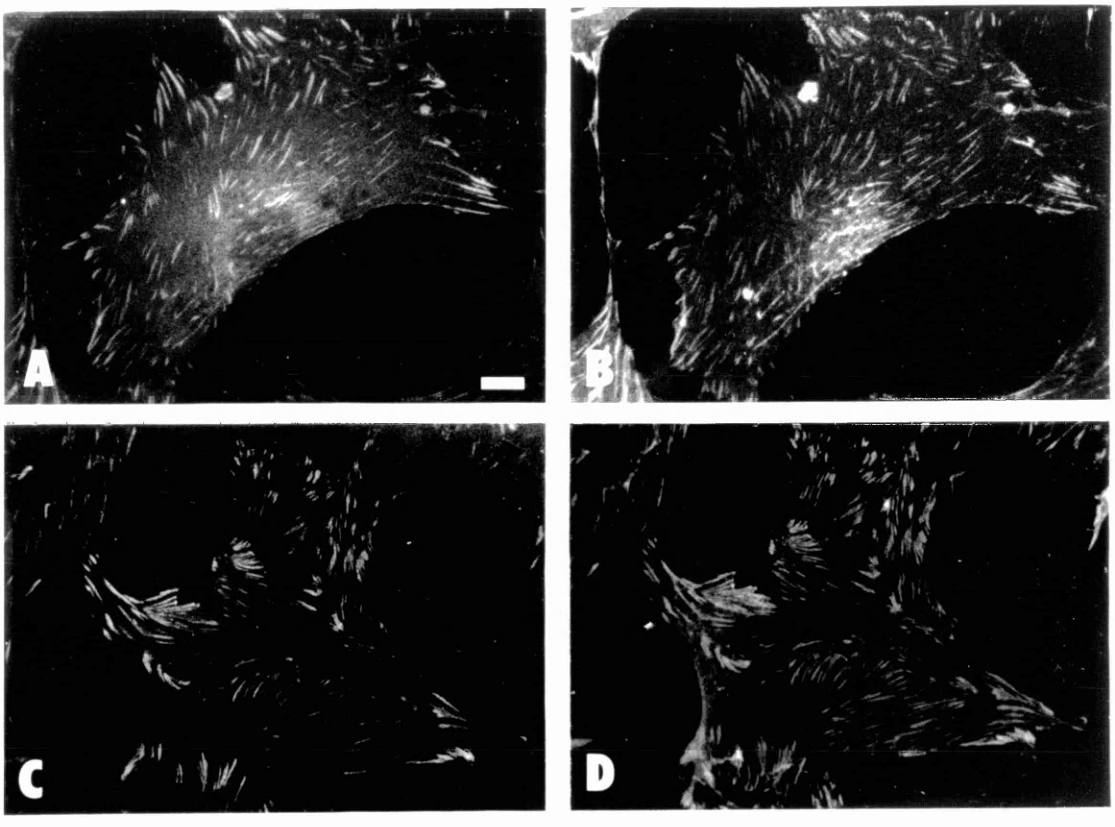


Figure 3.17. Localisation of paxillin in focal adhesions of VPMs.

Double immunolocalisation in CEFs (panels A and B) and in VPMs (panels C and D) of paxillin (panels A and C) and of the integrin β1 subunit (panels B and D). Samples were fixed and permeabilised as described in chapter 2. First antibodies were revealed with FITC anti-mouse Ig (panels A and C) and TRITC anti-rabbit Ig (panels B and D). Bar = 10 μm.

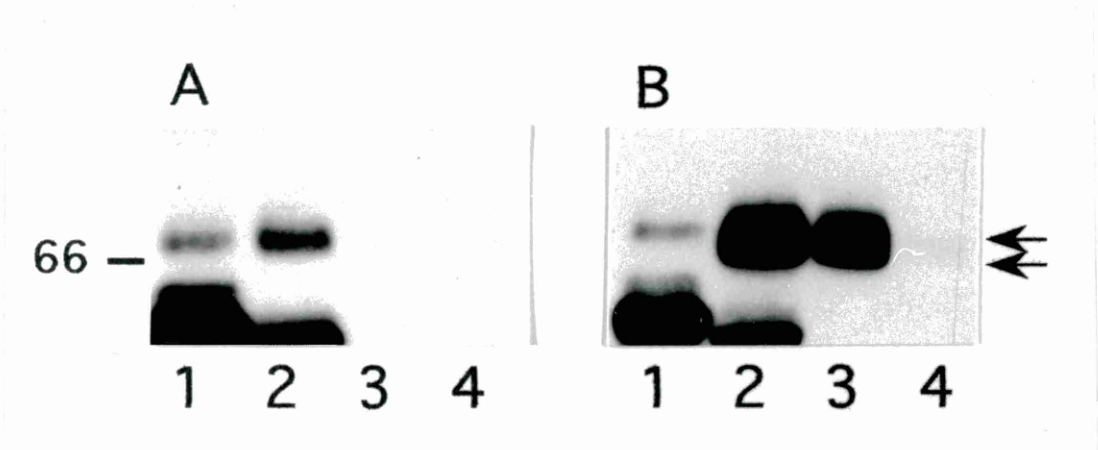


Figure 3.18. Tyrosine-phosphorylation of paxillin in CEFs.

200 μg of protein from CEF lysates were immunoprecipitated with anti-phosphotyrosine (lanes 1A and 1B) or with anti-paxillin (lanes 2A and 2B) antibodies. Equal amounts of the non-bound fractions from the anti-phosphotyrosine (lanes 3A and 3B) and anti-paxillin (lanes 4A and 4B) immunoprecipitations were loaded on the same gel. After blotting, part (A) of the blot was incubated with anti-phosphotyrosine antibodies, and part (B) was incubated with anti-paxillin antibodies. The 66 kDa molecular mass marker is indicated to the left of the blot. The two arrows to the right indicate the position of migration of the tyrosine-phosphorylated (upper arrow) and total (lower arrow) paxillin.

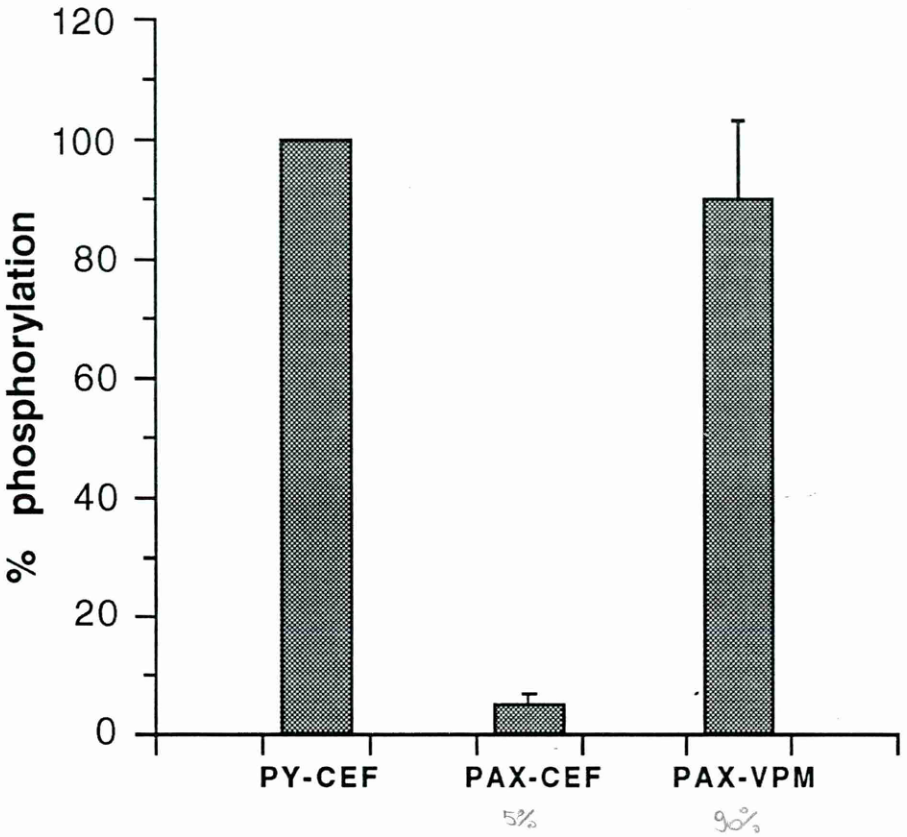


Figure 3.19. Comparison of the level of tyrosine-phosphorylation of paxillin in CEFs and in VPMs.

Bands from blots of experiments like the one described in Figure 18 were quantitated by densitometry, and the ratio between the values obtained from bands like those shown in lanes 1A and 1B of Figure 18 was used as 100% of tyrosine phosphorylation of paxillin (PY-CEF). From this value, the percentage of tyrosine-phosphorylated paxillin was calculated in CEF lysates (PAX-CEF), and in VPM lysates (PAX-VPM). The value for PAX-VPM was calculated from blots like those shown in Fig. 15 (lanes 2B and 2C). Each value represents the average from two different experiments. Bars = standard deviation.

3.3 PRODUCTION AND CHARACTERISATION OF mAbs RAISED AGAINST VPM PREPARATIONS

Although there is much known about the structure of focal adhesions, the exact architecture and the role of single polypeptides at these adhesive sites is still far from being clarified. Moreover, it is generally believed that some players and regulators of focal adhesions are still waiting to be discovered.

By using a combination of morphological and biochemical approach, in 3.1 and 3.2 we have demonstrated that VPM preparations preserve intact the focal adhesion complex and the connected actin cytoskeleton, and that they are enriched in some focal adhesion components and in tyrosine phosphorylated polypeptides. Therefore VPMs can be considered a subcellular fraction where focal adhesions are enriched when compared to the whole cell, and they represent an ideal starting material for a better characterisation of focal adhesion structure. With the aim of obtaining new important tools for functional studies about focal adhesions, and eventually identifying new components or regulators, we have injected VPM preparations into mice, for the production of mAbs. The clones obtained were screened by immunofluorescence for their localisation on VPMs, and some of them have been characterised. By using this approach we have obtained antibodies that were useful for studies of focal adhesion regulation and of the connected actin cytoskeleton, and were used for some of the experiments described in 3.4 and 3.5.

3.3.1 Preparation of material for immunisation

In order to prepare VPMs for mice injections, CEFs were cultured over night on fibronectin in the absence of serum to allow organisation of focal adhesions, and to minimise the production and secretion of other extracellular matrix, as well as the deposition of proteins from serum on the substrate. VPMs were prepared from CEFs, lysates were collected, and the material concentrated by acetone precipitation, as described in chapter 2. The quality of each preparation was controlled by analysing the pattern of polypeptides in lysates obtained from VPM preparations, in comparison with that obtained from intact CEFs. In Fig. 3.20 the same amount of protein from VPM preparations and from CEFs was loaded on a gel and processed for silver staining, as described in chapter 2. Arrows in lane 2 indicate some of the polypeptides enriched in VPM preparations. Arrowheads point to some examples of polypeptides that were lost or

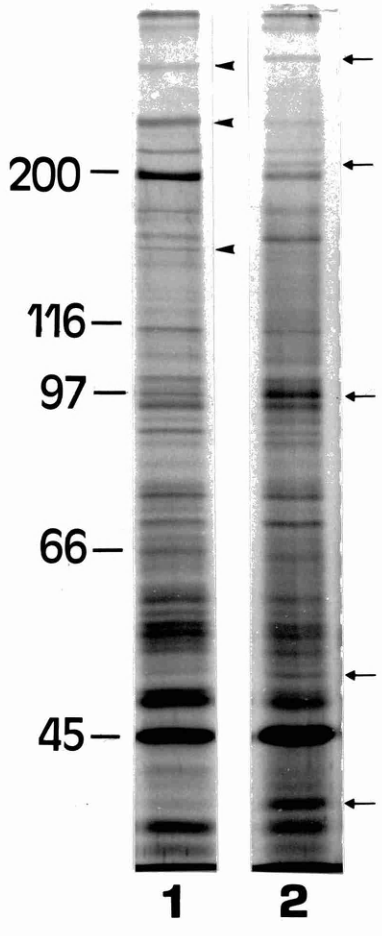


Figure 3.20. Analysis by silver staining of the patterns of polypeptides from CEF and VPM lysates

CEF (lane 1) and VPM lysates (lane 2) were prepared as described in chapter 2. 5 μg of each sample were loaded on a 6% acrylamide gel and processed for silver staining as described in chapter 2. Molecular mass markers are indicated on the left. Arrowheads in lane 1 indicate polypeptides enriched in CEFs compared to VPM preparations, while arrows in lane 2 indicate polypeptides enriched in VPM preparations.

decreased in amount in VPM preparations (lane 2), as compared to a total cell lysate (lane 1).

3.3.2 Mice immunisation, cell fusion, and screening

Each mouse was injected with 100 μg of protein, and at least 5 injections were performed, as detailed in chapter 2. Before fusion, sera from immunised animals were tested by immunofluorescence on VPM preparations together with the corresponding pre-immune sera. The resulting staining was strong and complex for all animals, and the pre-immune sera gave only a weak, non specific staining (not shown). Ag8 myeloma cells and lymphocytes from an immunised mouse were fused, hybridomas were selected, and their supernatants were screened by immunofluorescence, as described in chapter 2. 1882 wells out of 1920 contained growing cells, and were screened. Among these, 53 hybridomas were initially selected as positive, and some of these have been subcloned as described in chapter 2, and characterised. Fig. 3.21 shows the staining of some positives clones: S1E2 showed a microtubular staining on CEFs (panel A), and a similar but weaker staining on VPMs (not shown); T4F12 and AG1 showed nearly a diffused pattern on VPMs (panels B and C, respectively); H3A7 gave a punctate staining along stress fibers (panel D), and X1E8 stained homogeneously actin stress fibers (panel E), as confirmed by colocalisation with phalloidin (Fig. 3.22); M2D5 gave both a diffused and a fibrillar staining on the substrate, escluding VPMs, when permeabilisation with detergent was not performed (panel F). AF3 stained actin stress fibers and focal adhesions, and colocalised with β1 integrins in focal adhesions in several points (Fig. 3.23, arrowheads).

3.3.3 Ig class determination and biochemical characterisation

The Ig class of the selected clones was determined by ELISA, by using antibodies specific for μ or γ chains, as detailed in chapter 2. Fig. 3.24 shows the results obtained for the same clones shown in Fig. 3.20: S1E2, T4F12, AG1, H3A7, and M2D5 bound selectively to anti-γ chain antibodies, and were therefore IgG, while X1E8 and AF3 were IgM.

The same supernatants were characterised by western blotting on lysates from CEFs (Fig. 25): S1E2 gave a predominant band of 57 KD, compatible with the molecular weight of tubulins; T4F12 stained a single band of 40 KD; AG1 gave only some

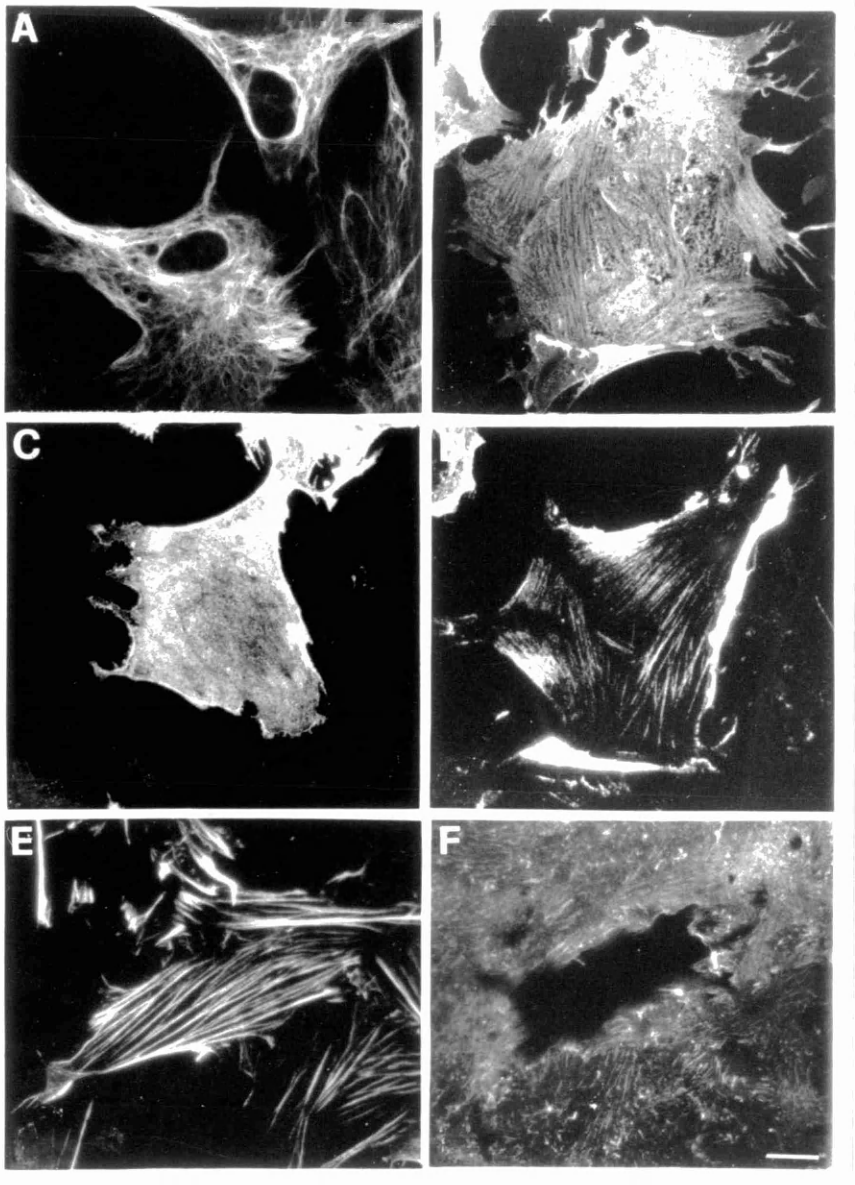


Figure 3.21. Localisation of the antigens recognised by some of the identified mAbs.

CEFs (panel A) or VPMs (panels B-F) were fixed, permeabilised (except for panel F), and immunostained as described in chapter 2, with the following hybridoma supernatants: S1E2 (panel A), T4F12 (panel B), AG1 (panel C), H3A7 (panel D), X1E8 (panel E), and M2D5 (panel F). Primary antibodies were revealed with FITC-conjugated donkey anti-mouse Ig. Bar = 10 μm.

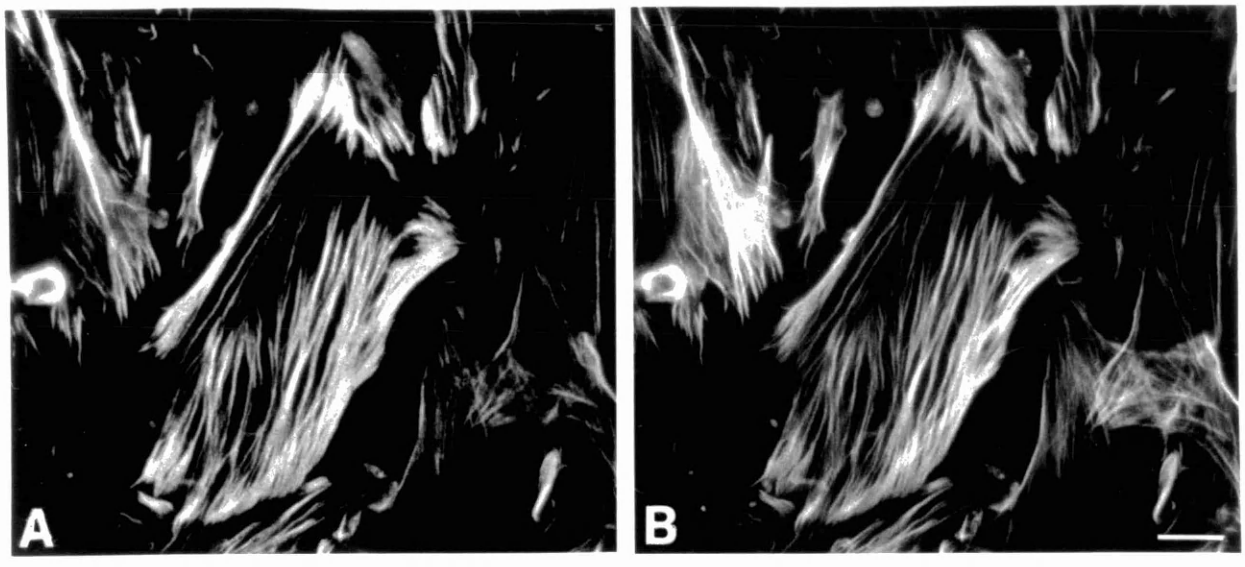


Figure 3.22. Colocalisation of the X1E8 antigen with actin on VPMs.

VPMs were prepared, fixed, and stained with X1E8 supernatant (panel A). The primary antibody was revealed with FITC-conjugated donkey anti-mouse Ig. RITC-phalloidin was used to stain actin stress fibers (panel B). Bar = 10 μm.

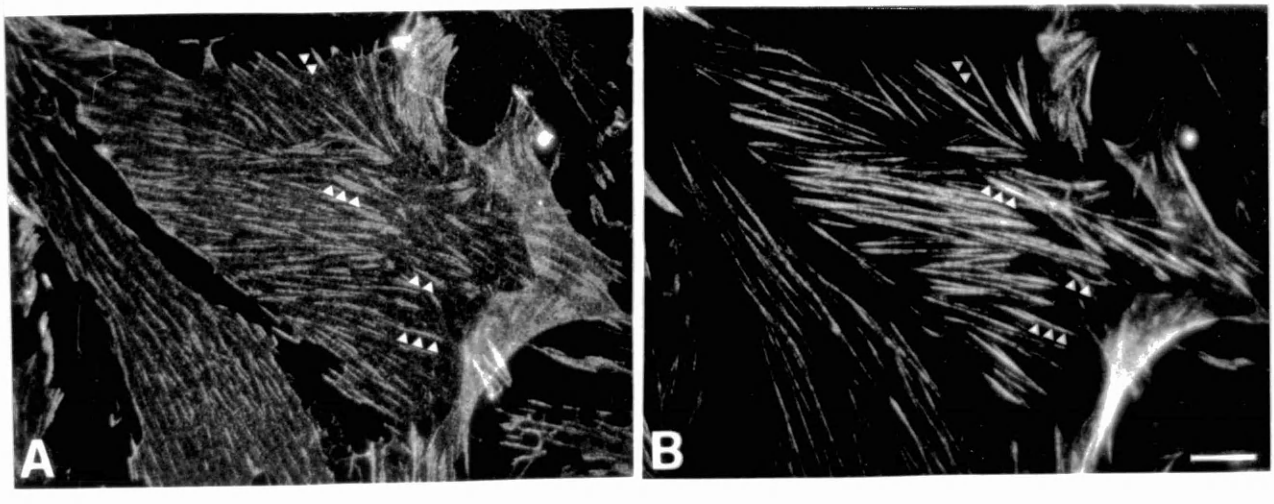


Figure 3.23. The AF3 antigen localises along actin stress fibers and focal adhesions on VPMs.

VPMs were prepared, fixed, and costained with β1 integrins (panel A), and AF3 supernatant (panel B), as described in chapter 2. Arrowheads indicate sites of colocalisation of the two antigens. Bar = 10 μm.

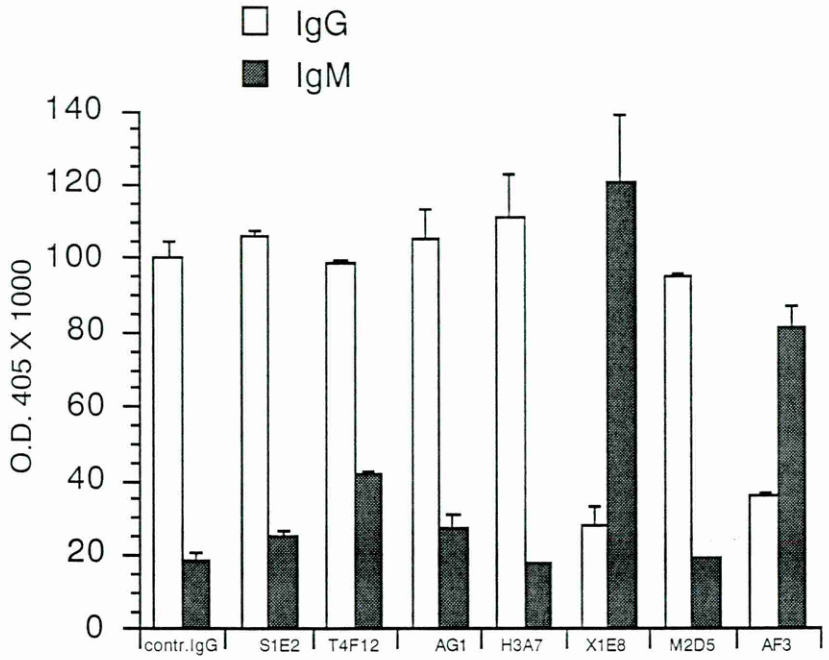


Figure 3.24. Ig class determination of some positives clones.

Wells from a 96-well plate were precoated with anti-γ (for IgG determination, □), or anti-μ (for IgM determination, ■) IgG. Ig from hybridoma supernatants were allowed to bind. Binding was quantified as described in chapter 2, and expressed as $O.D._{405}$ X 1000. MAb CSAT was used as positive control for IgG. Each sample was done in triplicate, and bars indicate the standard deviations.

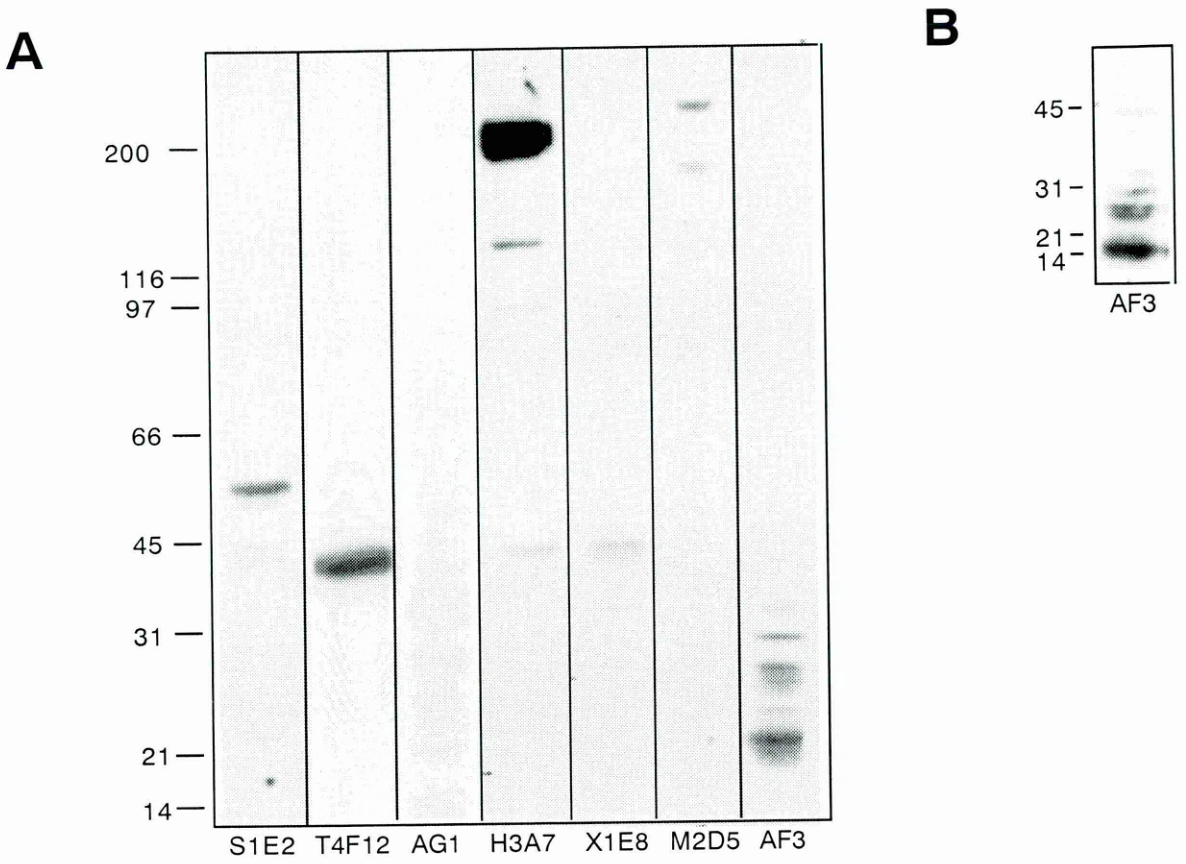


Figure 3.25. Western blotting of positive clones on CEF lysates.

100 µg protein from CEF lysates were loaded on each lane of a 5-13% gradient polyacrylamide gel. Each lane was analysed by western blotting with the indicated hybridoma supernatants (panel A). In panel B, the AF3 supernatant was diluted 1:50. Primary antibodies were revealed with ^{125}I-sheep anti-mouse Ig. Molecular mass markers are indicated on the left of each panel.

background and did not recognise any specific band on CEF lysate; H3A7 recognised a predominant band of 200 KD; X1E8 gave a faint band of the 45 KD. This result, together with the colocalisation of X1E8 antigen with phalloidin (Fig. 3.23), strongly indicated the correspondence of X1E8 antigen with actin. M2D5 gave a predominant band of 250 KD, a molecular weight compatible with that of extracellular matrix proteins, such as fibronectin; while AF3 stained a series of bands between 31 and 14 KD, and the pattern did not become simpler by incubation of the filters with higher dilutions of the hybridoma supernatant (Fig. 3.25 B).

To look at the expression of the antigens in neural tissue, the hybridoma supernatants were tested by western blotting on CEF lysates, together with lysates obtained from E13 chick embryonic brain. The results shown in Fig. 3.26 indicated that only S1E2 and X1E8 recognised antigens present both in brain and fibroblasts, as expected from broadly expressed and conserved proteins such as tubulin and actin, while all the other clones recognised antigens present only in fibroblasts.

S1E2 antigen was further characterised by comparing its exact molecular weight with that of chicken α and ß-tubulin. In Fig. 3.27 A, CEF lysates were analysed by western blotting with S1E2 supernatant (lanes 2 and 4), and with anti-α-tubulin (lane 1) or anti-ß-tubulin (lane 3) antibodies: S1E2 antigen comigrated exactly with chicken α-tubulin, while it migrated slightly slower than β-tubulin. These results, together with the tubular staining of S1E2 in CEFs (see Fig. 3.21 A), suggest the correspondence of S1E2 antigen with α-tubulin.

In order to characterise M2D5 antigen, various extracellular matrix proteins were loaded on a gel and analysed by western blotting with M2D5 mAb (Fig. 3.27 B). M2D5 recognised the purified fibronectin (lane 1), while it did not recognise collagen I (lane 2) or tenascin C (lane 3). As shown in Fig. 3.25, M2D5 recognised a 250 KD polypeptide in CEFs, and it recognised a band of the same molecular weight in the supernatant from CEFs cultures (lane 5). This result indicates that the antigen recognised by the M2D5 mAb corresponds to a secreted protein.

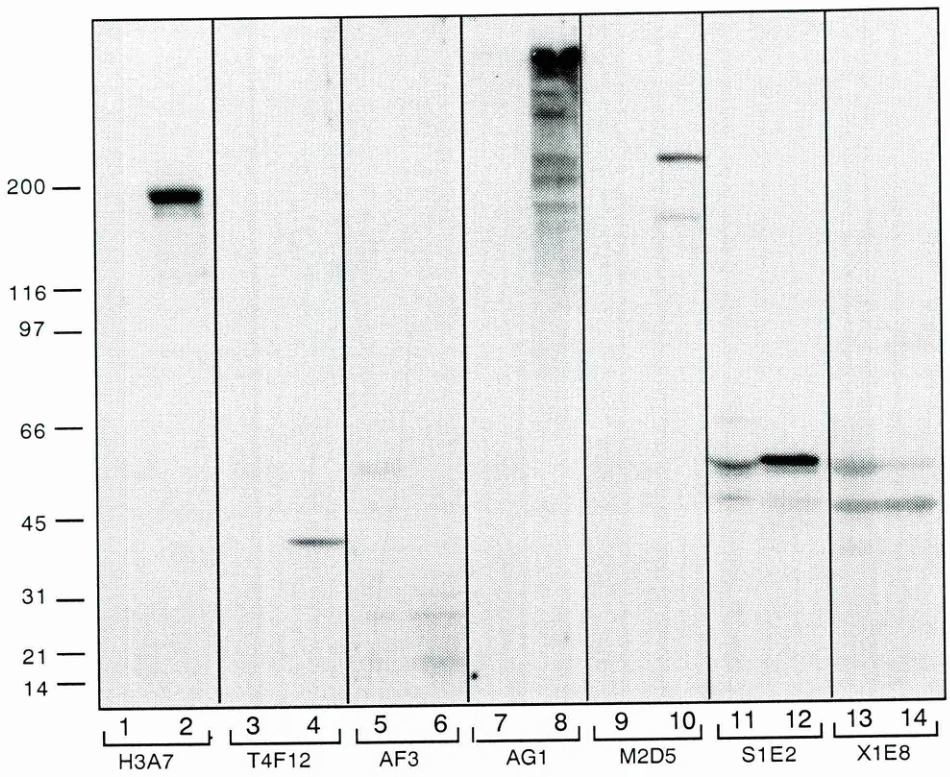


Figure 3.26. Analysis of the expression of the antigens in chick embryonic brain

100 μg protein from E13 embryonic chick brain lysate (odd lanes) and from CEF lysate (even lanes) were analysed by western blotting on a 5-13% gradient gel, by using the indicated hybridoma supernatants as primary antibodies, as described in chapter 2. Molecular mass markers are indicated on the left.

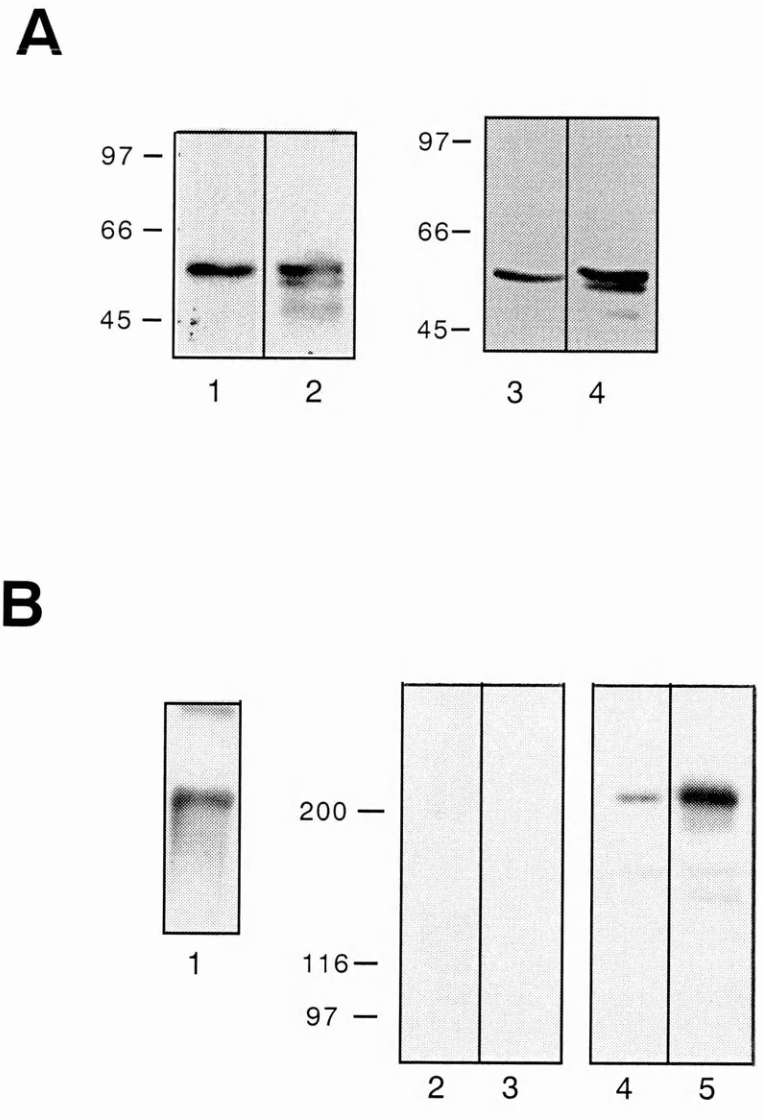


Figure 3.27. The S1E8 antigen comigrates with α-tubulin, and M2D5 specifically recognises fibronectin.

Panel A: 100 μg CEF lysate were loaded on each lane of a 5-13% acrylamide gel, and western blotting was performed as described in chapter 2, by using S1E8 hybridoma supernatant (lanes 2 and 4), and anti-α-tubulin (lane 1) or anti-β-tubulin (lane 3) mAbs. Panel B: 2 μg purified fibronectin (lane 1), collagen I (lane 2), or tenascin C (lane 3), and 50 μg CEF lysate (lane 4), or supernatant from CEF cultures (lane 5) were loaded on a 5% acrylamide gel, and analysed by western blotting as described in chapter 2, by using M2D5 supernatant as primary antibody. Molecular mass markers are indicated on the left.

3.4 [Ca^{2+}]-REGULATED β1 INTEGRINS FUNCTION AND DISTRIBUTION ON VPMs

Assembly and modulation of focal adhesions during dynamic adhesive processes are poorly understood, and the precise role of numerous proteins colocalising with these structures is not estabilished yet. The set up of a "cell-free" system would be of great advantage for a better understanding of the sequence of molecular events leading to focal adhesion organisation, and to the exploration of focal adhesion regulation and dynamics. In the previous paragraphs, I have described the characterisation of detergent-free VPMs obtained from adherent CEFs. I have shown that VPMs contain well-structured focal adhesions and stress fibers, as detected both by morphological and biochemical criteria. Two important advantages make this system useful for functional studies about focal adhesion dynamics: the preservation of the adhesive receptors within the natural lipidic enviroment (i.e., the adherent portion of the plasma membrane of cells spread on ECM), and the accessibility to the cytoplasmic side of the adhesive membrane, without need for detergents, that may affect the enviroment of the adhesive receptors. By using this system, I show that it is possible to reversibly modulate integrin function and distribution within VPMs. The requirements for such a modulation will be investigated, with a particular attention to the connection with focal adhesions components and the actin cytoskeleton.

3.4.1 Regulation of β1 integrin distribution by calcium under cell-free conditions

I have utilised VPMs prepared from adherent fibroblasts with the aim of setting up an *in vitro* system to study the regulation of focal adhesions and integrin function under cell-free conditions. For this purpose, I have treated VPMs under different experimental conditions to identify parameters that would affect integrin distribution and/or function *in vitro*. At the end of each experiment, cells were fixed for analysis by immunofluorescence, as described in chapter 2.

Among the experimental parameters examined were ionic composition, ionic strength, divalent cations, pH, and temperature. Surprisingly, preliminary analysis showed that VPMs prepared from CEFs in the presence of divalent cations appeared well preserved also after several hours of incubation at 37°C, as detected by staining with the lipophilic dye $DiIC_{16}$ (not shown). I then utilised immunofluorescence to look for

conditions that would affect integrin distribution upon incubation of VPMs at 37°C. Control, untreated samples were obtained by fixing the VPMs immediately after preparation on ice. Staining of control VPMs with the polyclonal antibody β1-cyto showed that the receptors were localised both into focal adhesions, and diffuse throughout the ventral surface of the adherent fibroblasts (Fig. 3.28A). We then tested the effect of the incubation of the VPMs at 37°C for 15 minutes in different buffers. Among the parameters analysed, variations in $[Ca^{2+}]$ in the presence of 2.5 mM $MgCl_2$ showed interesting effects on the distribution of the β1 receptors *in vitro*. When VPMs were incubated for 15 min at 37°C in a buffer containing $[Ca^{2+}]$s equal or above 10 μM, β1 integrins appeared predominantly diffuse on the membrane, and their distribution became more homogeneous after incubation at 37°C with 1 mM free Ca^{2+} (Fig. 3.28C). The diffusion of the integrin β1 subunits at high $[Ca^{2+}]$ was not caused by disassembly of the α/β heterodimers, since biochemical analysis showed coprecipitation of the α6 subunits with β1 under these conditions (Fig. 3.29). The β1 receptors showed a dramatically different distribution when VPMs were incubated at low $[Ca^{2+}]$. In fact, after incubation at 37°C for 15 min in LCB (containing 50 nM free Ca^{2+}), β1 integrins were found concentrated along fibrillar structures (Fig. 3.28E), which were generally thinner and longer compared to focal adhesions (compare Fig. 3.28E with Fig. 3.28A). In this situation, the diffuse integrin staining observed in control VPMs disappeared, suggesting that all β1 integrins had redistributed into the fibrillar structures. It should be emphasised though that the fibrillar distribution of integrins observed at low $[Ca^{2+}]$ is different from that of typical focal adhesions observed in whole cells, or in untreated VPMs (compare Fig. 3.28E with 3.28A). The same results were observed when control VPMs or samples treated with high or low $[Ca^{2+}]$ were analysed by immunofluorescence with the CSAT monoclonal antibody, directed against a distinct epitope of the β1 subunit (Fig. 3.28B, D, and F). These findings strongly indicate that the distinct patterns observed are due to actual changes in receptor distribution, rather than to the inability of a specific antibody to fully recognise the β1 antigen under different experimental conditions. Although 15 min incubation was utilised as standard experimental condition, both high and low $[Ca^{2+}]$-induced effects were observed already after 3 min at 37°C, while incubation at 0°C under different $[Ca^{2+}]$s did not affect integrin distribution (not shown).

To investigate the reversibility of the $[Ca^{2+}]$-mediated effects on β1 distribution, VPMs treated for 15 min at 37°C in HCB, were incubated for an additional 15 min in LCB. Under these conditions, β1 integrins that were diffused on the membrane after

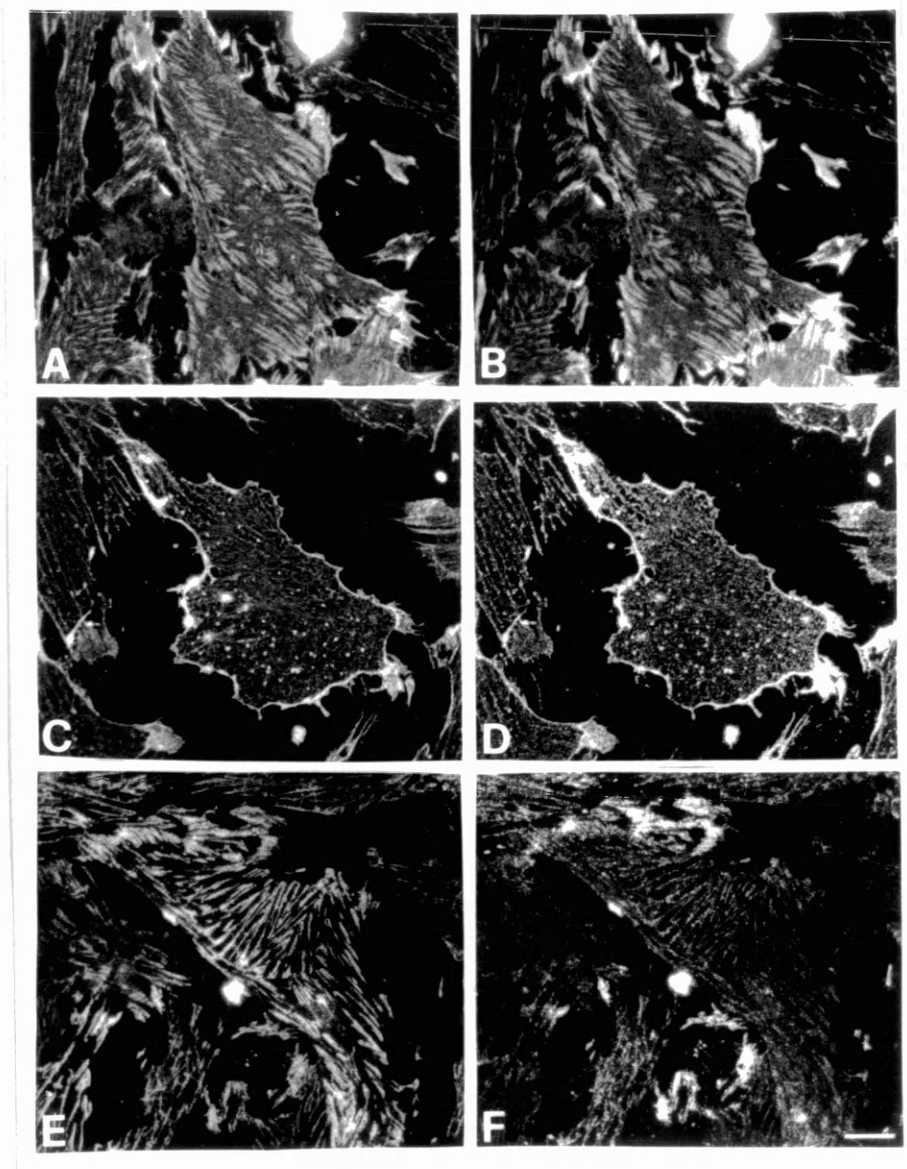


Figure 3.28. Calcium-dependent redistribution of β1 integrins in VPMs.

Adherent CEFs were incubated 20 min at room temperature with 30 μg/ml CSAT mAb (panels B, D and F), before preparation of VPMs. VPMs were fixed after preparation on ice (panels A, B), or after incubation for 15 min at 37°C in HCB (panels C, D), or in LCB (panels E, F). VPMs were then processed for immunofluorescence with the β1-cyto polyclonal antibody (panels A, C and E). Primary antibodies were revealed by FITC-conjugated anti-rabbit IgG and TRITC-conjugated anti-mouse IgG, respectively. The same fields are shown in panels A and B, C and D, and E and F. Bar = 10 μm.

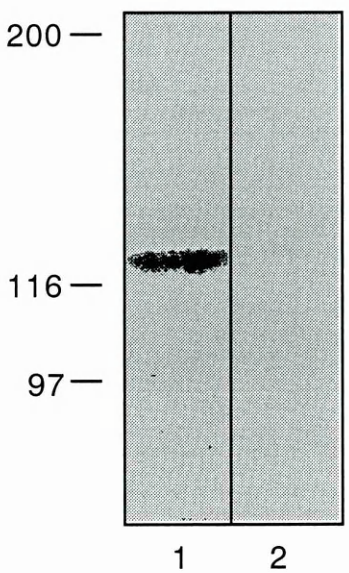


Figure 3.29. The association between β1 and α6 subunits is mantained after treatment of VPMs with high [Ca^{2+}].

VPMs from adherent CEFs were incubated in HCB for 15 min at 37°C, and lysates were obtained as described in chapter 2. Immunoprecipitation was performed by using an anti-β1 integrin serum. The immunoprecipitate (lane 1), and half of the unbound fraction (lane 2), were loaded on a 5% acrylamide gel and analysed by western blotting with the α6-EX pAb, recognising the α6 integrin subunit. Molecular mass markers are indicated on the left.

treatment in HCB (Fig. 3.30A), were now found concentrated into fibrillar structures (Fig. 3.30B). In contrast, β1 receptors concentrated into fibrillar structures by 15 min incubation at 37°C in LCB were not able to diffuse when VPMs were incubated for further 15 min in HCB (Fig. 3.30C). These results show that high $[Ca^{2+}]$-induced β1 integrins diffusion is quasi-reversible, since it can be affected by low $[Ca^{2+}]$. On the other hand, low $[Ca^{2+}]$ treatment somehow irreversibly locks β1 integrins into the fibrillar pattern.

To control whether the inability of the receptor concentrated into fibrillar structures in LCB to diffuse again after high $[Ca^{2+}]$ treatment was due to disruption of the membranes, we used the lipophilic fluorescent dye $DiIC_{16}$ to stain VPMs treated at 37°C for 15 min in LCB followed by 15 min incubation in HCB (Fig. 3.30D). Membrane integrity was not affected by this treatments. These findings strongly indicate that the distinct patterns observed at different $[Ca^{2+}]$s, together with the inability of the receptor to reverse its distribution from low to high $[Ca^{2+}]$, are due to actual changes in receptor distribution within the lipid bilayer, rather than to a modification of the structure of the plasma membrane.

3.4.2 $[Ca^{2+}]$ regulates the distribution of β1 integrins on VPMs by affecting the affinity for the extracellular ligand

Integrin receptors may be expressed in different activation states on the surface of the cell. We tested the hypothesis that the different $[Ca^{2+}]$s were affecting β1 integrin distribution by modulating the affinity of the receptors for their ECM ligands. For this purpose we compared the distribution of the total population of β1 receptors, using the antibody β1-cyto, with the distribution obtained by staining with the mAb TASC, which recognises activated, high affinity β1 receptors (Neugebauer and Reichardt, 1991). In control VPMs, the β1-cyto antibody recognised the receptors concentrated into focal adhesions, as well as those diffused throughout the membrane (Fig. 3.31A), while the mAb TASC only recognised the receptors localised in focal adhesions (Fig. 3.31B). Furthermore, TASC did not recognise the diffuse β1 receptors in VPMs treated with HCB (compare Fig. 3.31D with 3.31C), while it was able to recognise the LCB-induced fibrillar pattern (compare Fig. 3.31F with 3.31E). These results show that under cell-free conditions, the $[Ca^{2+}]$ is able to affect β1 integrins distribution, possibly by regulating the ability of the receptors to recognise their extracellular ligands.

CEFs in culture are known to produce their own ECM, which they deposit and organise

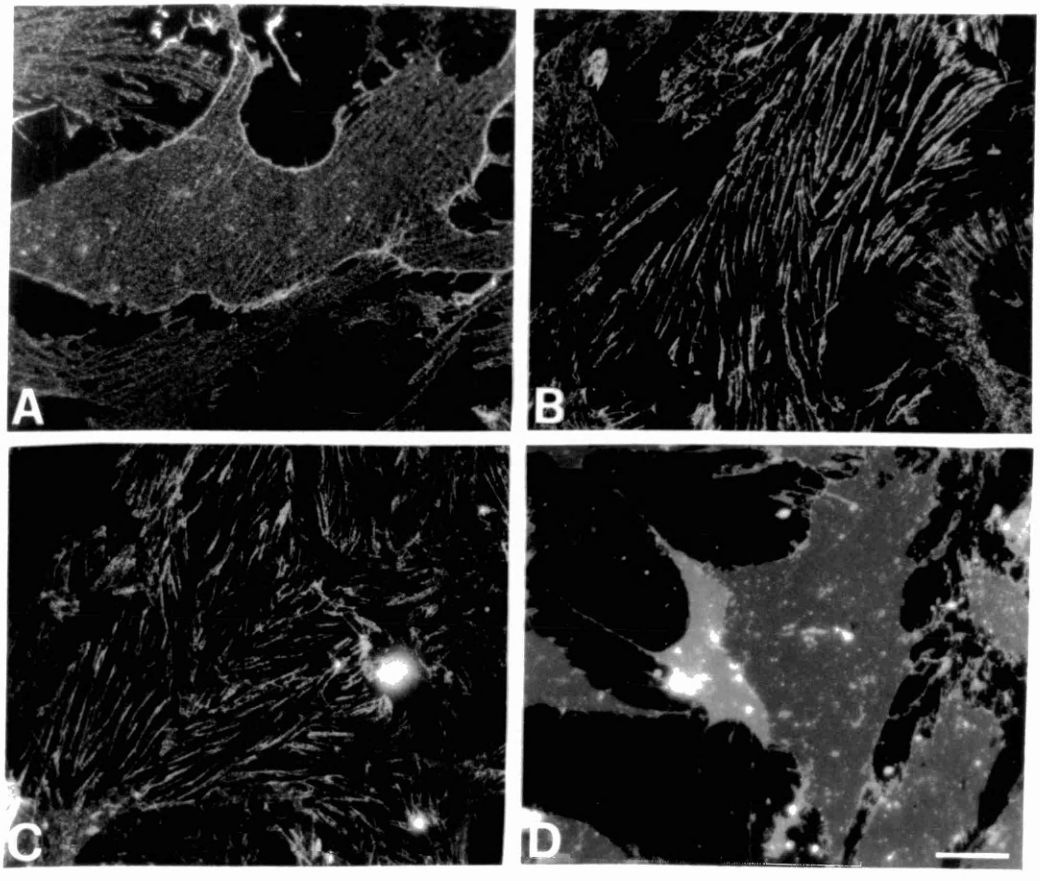


Figure 3.30. Reversibility of high [Ca^{2+}]-induced β1 integrins redistribution on VPMs.

VPMs from adherent CEFs were incubated in HCB for 15 min at 37°C (panel A), in HCB followed by 15 min at 37°C in LCB (panel B), or in LCB followed by 15 min at 37°C in HCB (panels C and D). In panel D, VPMs were stained with the lipophilic carbocyanine dye $DiIC_{16}$. After fixation, VPMs in panels A, B and C were processed for immunofluorescence with the β1-cyto polyclonal antibody, revealed by a following incubation with FITC-conjugated anti-rabbit IgG. Bar = 10 μm.

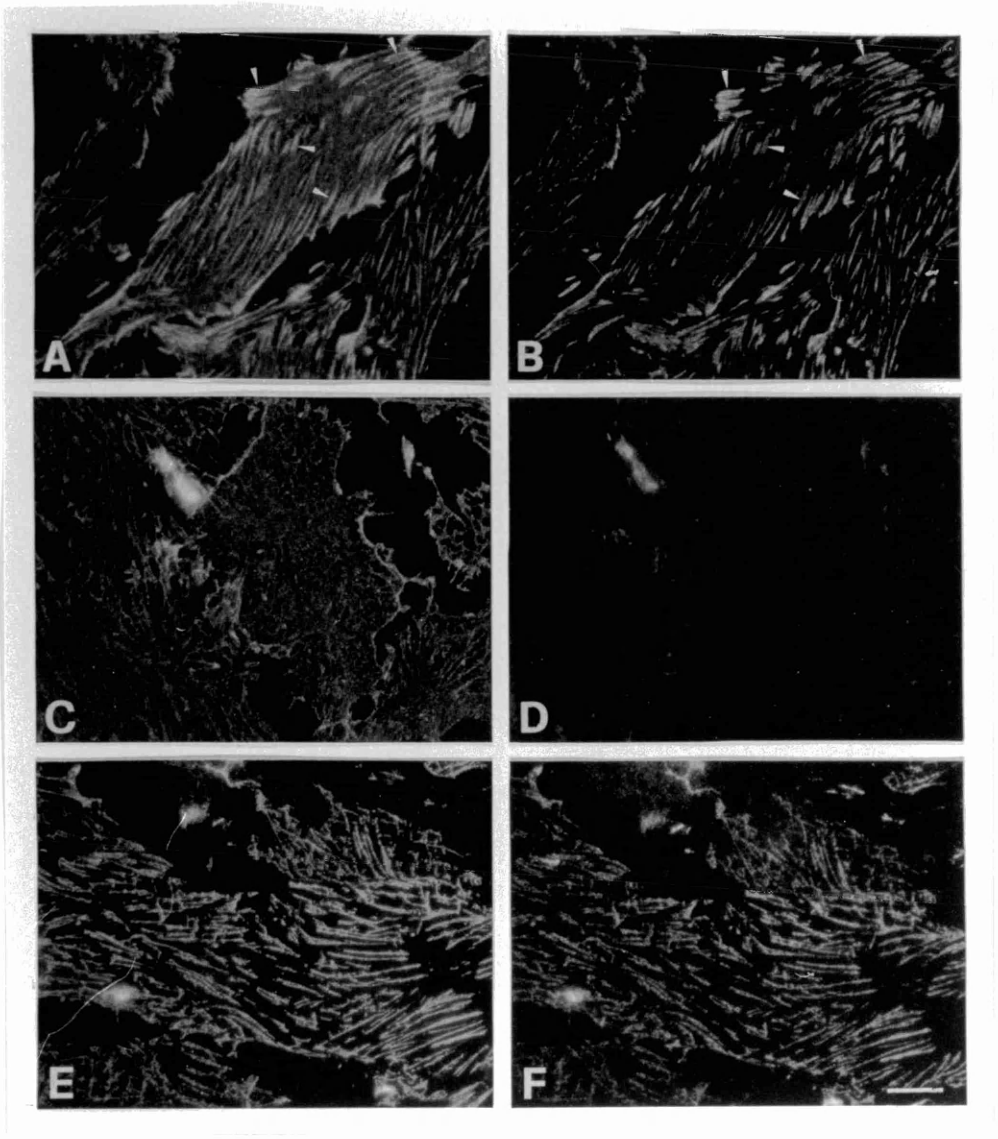


Figure 3.31. The mAb TASC only recognises β1 integrins localised in focal adhesions and along ECM fibrils.

VPMs were incubated with 20 μg/ml of the anti-integrin β1 TASC mAb for 20 min at 0°C (panels B, D and F), and immediately fixed (panels A and B), or incubated for 15 min at 37°C in HCB (panels C, D), or in LCB (panels E, F), before fixation. VPMs were then processed for immunofluorescence with the β1-cyto polyclonal antibody (panels A, C and E). Primary antibodies were revealed by FITC-conjugated anti-rabbit IgG and TRITC-conjugated anti-mouse IgG, respectively. The same fields are shown in panels A and B, in panels C and D, and in panels E and F. Arrowheads in panels A and B point to focal adhesions, where colocalisation of β1-cyto and TASC staining is visible. Bar = 10 μm.

on the substrate. The low [Ca^{2+}]-induced redistribution of β1 integrins into fibrillar structures suggested a possible relocalisation of the receptors along ECM fibrils underlying the VPMs. We therefore analysed the distribution of the receptors and of ECM in control and experimentally treated VPMs. In control VPMs fixed after preparation at 0°C, ECM fibrils, stained by the mAb M2D5 recognising chicken fibronectin (see pharagraph 3.3 for M2D5 characterisation), colocalised only partially with β1-positive focal adhesions (Fig. 3.32A and B, arrowheads). After incubation for 15 min at 37°C in HCB, β1 integrins were predominantly diffuse on the membrane (Figure 3.32C), while ECM fibrils could still be observed (Figure 3.31D). Incubation for 15 min at 37°C in LCB induced a redistribution of β1 integrins to sites which strikingly corresponded to the ECM fibrils (Figure 3.31E, F, arrowheads).

We utilised immunoelectron microscopy to obtain more detailed information on the nature of the fibrillar structures where β1 integrins localise at low [Ca^{2+}]. Figure 3.33 shows different "en face" views of VPMs treated with LCB. Bundles of actin filaments were visible as electron dense structures that were abundantly decorated both by the mAb X1E8, characterised in pharagraph 3.3 (Fig. 3.33A, small gold particles), and by a polyclonal anti-actin antibody (Fig. 3.33B, large gold particles). β1 integrins were concentrated along fibrils, which sometimes ran parallel to the actin bundles (Fig. 3.33A, large gold particles), but most of the times the receptors were clearly separated from them (Fig. 3.33B, small gold particles). These structures correspond to ECM fibrils, since they could be stained with the mAb M2D5 recognising fibronectin (Figure 3.33C, small gold particles), and were often heavily decorated by anti-integrin β1 antibodies (Figure 3.33C, large gold particles). Ultrastructural analysis of VPMs incubated in HCB showed a diffuse distribution of the β1 receptors (Fig. 3.34, large gold particles), which had no obvious colocalisation with ECM fibrils (Fig. 3.34, small gold particles), confirming the data obtained by immunofluorescence. As expected from the diffuse ECM staining observed around fibrils by immunofluorescence (see Fig. 3.32B, D, and F), diffuse ECM staining could be observed also at the ultrastructural level in VPMs treated with both LCB and HCB (small gold particles in Fig. 3.33C, and 3.34, respectively).

These results show that the fibrillar structures decorated by integrins upon incubation at low [Ca^{2+}] correspond to sites of fibrillar accumulation of ECM, which are distinct from actin stress fibers.

Interestingly, when CEFs were cultured on a non fibrillar substrate such as laminin, under conditions that inhibit production of ECM (see MATERIALS AND

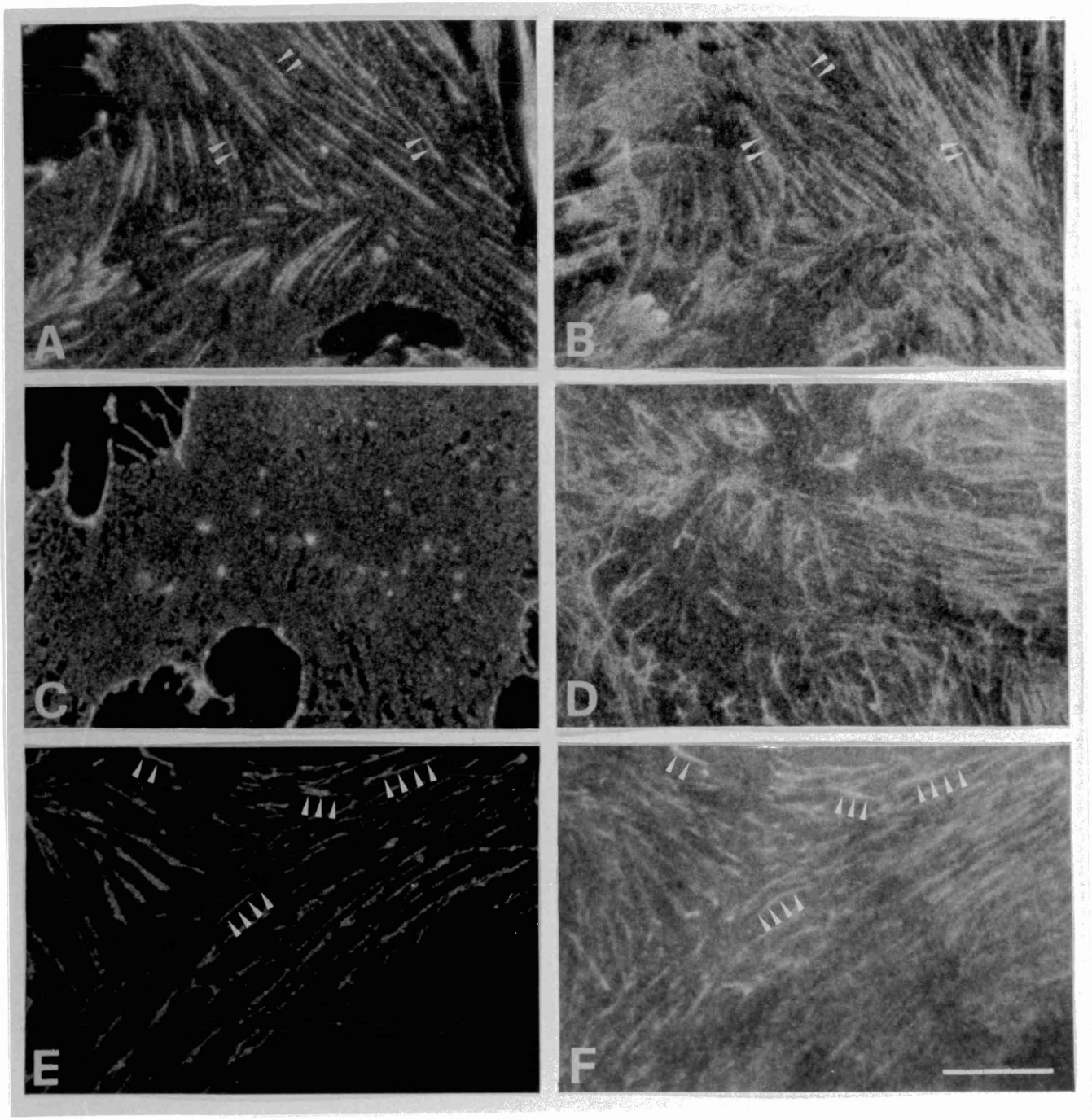


Figure 3.32. Localisation of β1 integrins and ECM fibrils after treatment of VPMs at different calcium concentrations.

VPMs from CEFs were fixed immediately after preparation on ice (panels A and B), after incubation for 15 min at 37°C in HCB (panels C and D), or after incubation for 15 min at 37°C in LCB (panels E and F). Fixed VPMs were processed for immunofluorescence using the β1-cyto antibody (panels A, C, and E), and the M2D5 mAb recognising fibronectin (panels B, D, and F). Primary antibodies were revealed by FITC-conjugated anti-rabbit IgG, and TRITC-conjugated anti-mouse IgG. The same fields are shown in panels A and B, C and D, E and F. Arrowheads in panels A and B, and in panels E and F point to sites where colocalisation of integrins with ECM fibrils is visible. Bar = 10 μm.

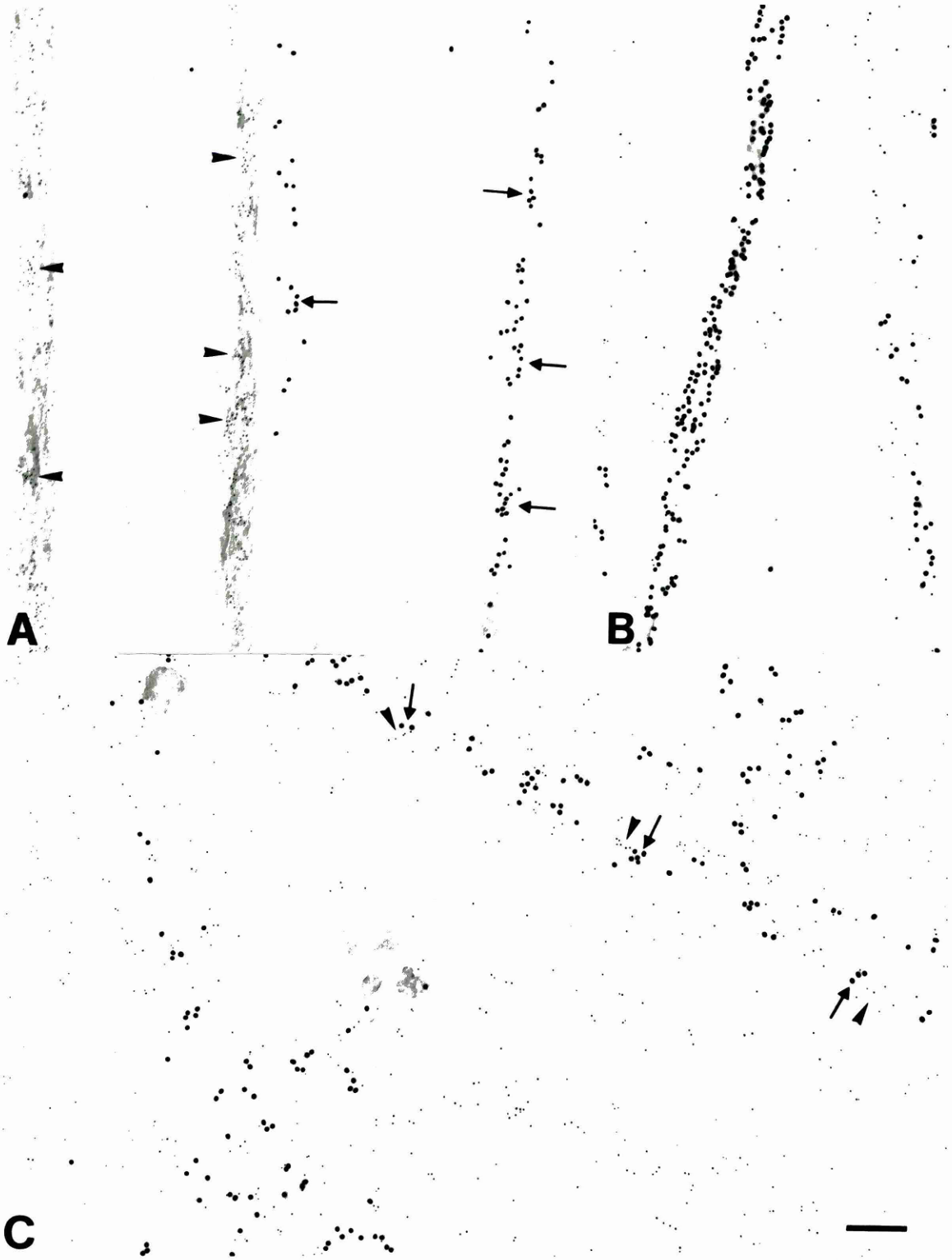


Figure 3.33. Ultrastructural localisation of β1 integrins along ECM fibrils at low [Ca^{2+}].

VPMs were incubated for 15 min at 37°C in LCB, and processed for immunoelectron microscopy as described in chapter 2. Panel A: double labelling with the β1-cyto antibody (large, 15 nm gold, arrows), and the X1E8 mAb decorating actin stress fibers (small, 6 nm gold, arrowheads); Panel B: double labelling with the anti-β1 TASC mAb (small, 6 nm gold), and the AAL20 polyclonal antibody against actin, decorating actin stress fibers (large, 15 nm gold); Panel C: fibrillar structures are labelled both by the β1-cyto antibody (large gold particles) and by the mAb M2D5 recognising ECM (small gold particles). In panel C, a few examples are shown of colocalisation of β1 integrins (arrows), with ECM (arrowheads) along fibrils. Bar = 200 nm.

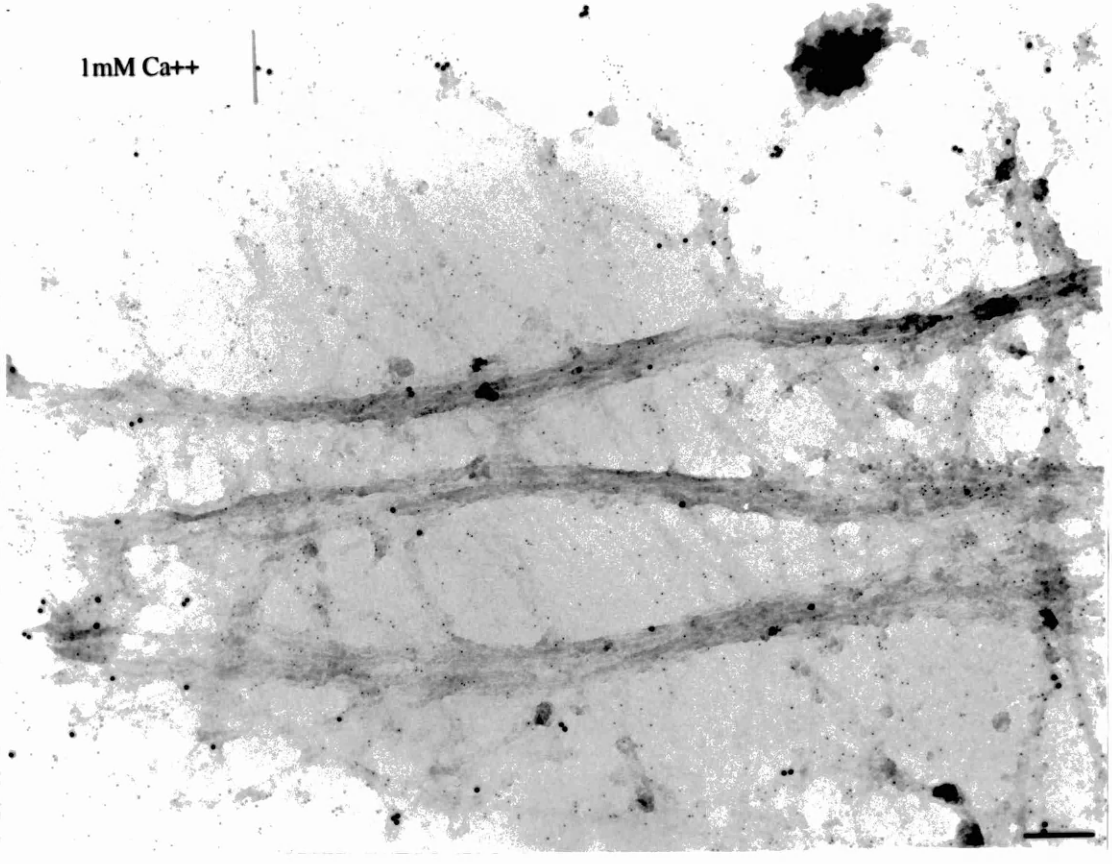


Figure 3.34. Ultrastructural localisation of β1 integrins and ECM fibrils at high [Ca^{2+}].

VPMs were incubated for 15 min at 37°C in HCB, and processed for immunoelectron microscopy as described in chapter 2. Diffused β1 integrins are labelled with the β1-cyto antibody (large, 15 nm gold, arrows), while ECM is labelled with the mAb M2D5 recognising fibronectin (small gold particles). Bar = 200 nm.

METHODS for details), β1 integrins which were concentrated in focal adhesions and diffused in control VPMs (Fig. 3.35A), gave a diffuse pattern after treatment with both high (Fig. 3.35C), and low [Ca^{2+}]s (Fig. 3.35E). Under these condotions, the mAb TASC was still able to recognise only the receptors concentrated in focal adhesions in the control situation (Fig.3.35B); it was unable to recognise the receptors diffused in HCB (Fig. 3.35D), while it was able to recognise the receptor diffused in LCB (Fig. 3.34F).

All together these results suggest that the pattern of distribution of the high-affinity (TASC-positive) integrins is a consequence of the activation of the receptors induced by low [Ca^{2+}], and depends on the organisation of the available ECM.

3.4.3 β1 integrins distribution can be altered by mAbs affecting receptor function

We investigated whether mAbs known to affect β1 integrin function were able to induce receptor redistribution *in vitro*. Incubation of VPMs for 15 min at 37°C in HCB in the presence of the stimumatory anti-β1 integrin mAb TASC was able to prevent high [Ca^{2+}]-induced β1 integrins diffusion, and to cause receptor clustering along ECM (Fig. 3.36A and B). This effect was similar to that observed after incubation of VPMs in LCB (Fig. 3.28E). As already shown in Fig. 3.30C, the effect of low [Ca^{2+}] on receptor distribution could not be reversed by incubation in HCB (Fig. 3.36C). On the other hand, when VPMs which had been incubated at low [Ca^{2+}] were further incubated at high [Ca^{2+}] in the presence of the function-blocking mAb CSAT, receptor clustering induced by low [Ca^{2+}] could be partially reversed (Fig. 3.36D), since a clear diffuse staining for integrins could be observed together with the fibrillar pattern. This result indicates that the presence of the function-blocking antibody can disrupt low [Ca^{2+}]-mediated integrin distribution, probably by interfering with receptor-ligand binding. Furthermore, the low [Ca^{2+}]-induced aggregation of receptors in VPMs previously treated with HCB (Fig. 3.36E), was largely prevented by incubating HCB-treated VPMs with LCB in the presence of the CSAT mAb (Fig. 3.36F).

These results confirm the idea that high [Ca^{2+}]-induced receptor diffusion within ventral surface of the cell may be explained by inhibition of integrin function, while low [Ca^{2+}]-induced receptor concentration along ECM fibrils may be explained by receptor activation. These data also point out that the effects of function-affecting antibodies on receptor distribution/activity are dominant over the [Ca^{2+}]-induced effects under our

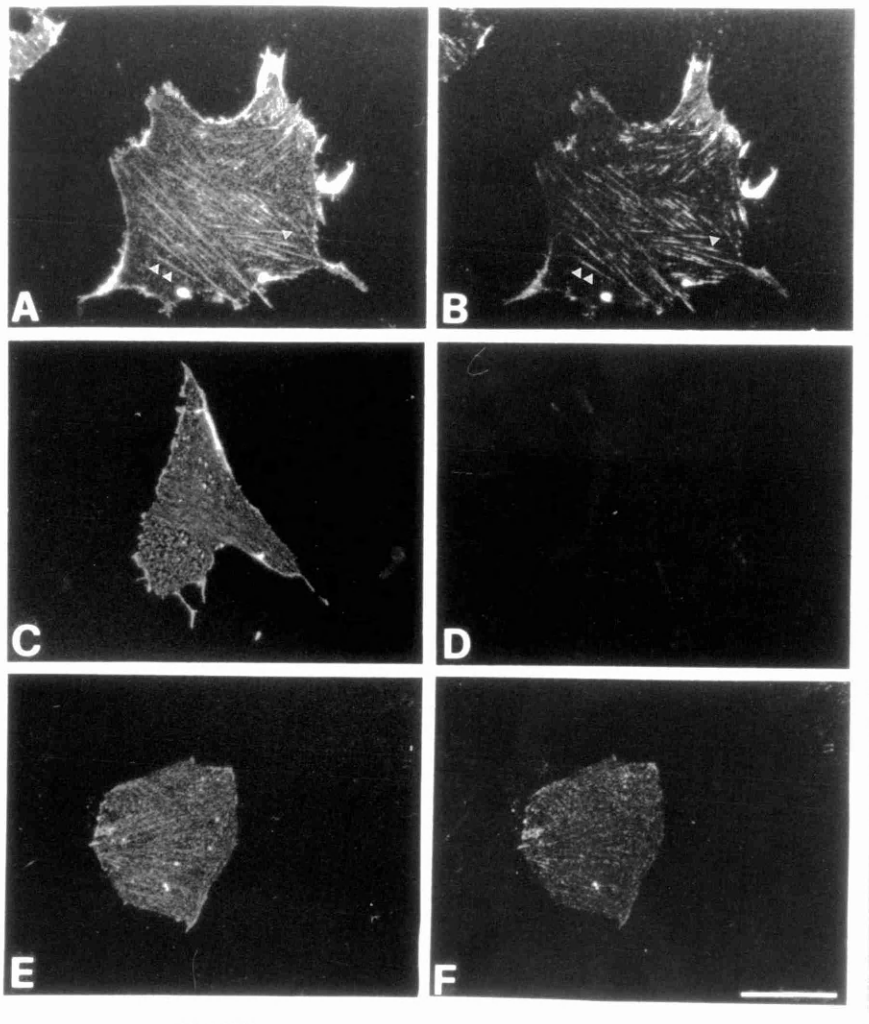


Figure 3.35. β1 integrins distribution after treatment at different calcium concentrations of VPMs from CEFs adherent to laminin.

VPMs were prepared from CEFs cultured for 3 h on laminin, under the conditions described in chapter 2, incubated with 20 μg/ml of the anti-integrin β1 TASC mAb for 20 min at 0°C (panels B, D and F), and immediately fixed (panels A and B), or incubated for 15 min at 37°C in HCB (panels C, D), or in LCB (panels E, F), before fixation. VPMs were then processed for immunofluorescence with the β1-cyto polyclonal antibody (panels A, C and E). Primary antibodies were revealed by FITC-conjugated anti-rabbit IgG and TRITC-conjugated anti-mouse IgG, respectively. The same fields are shown in panels A and B, in panels C and D, and in panels E and F. Arrowheads in panels A and B point to focal adhesions, where colocalisation of β1-cyto and TASC staining is visible. Bar = 10 μm.

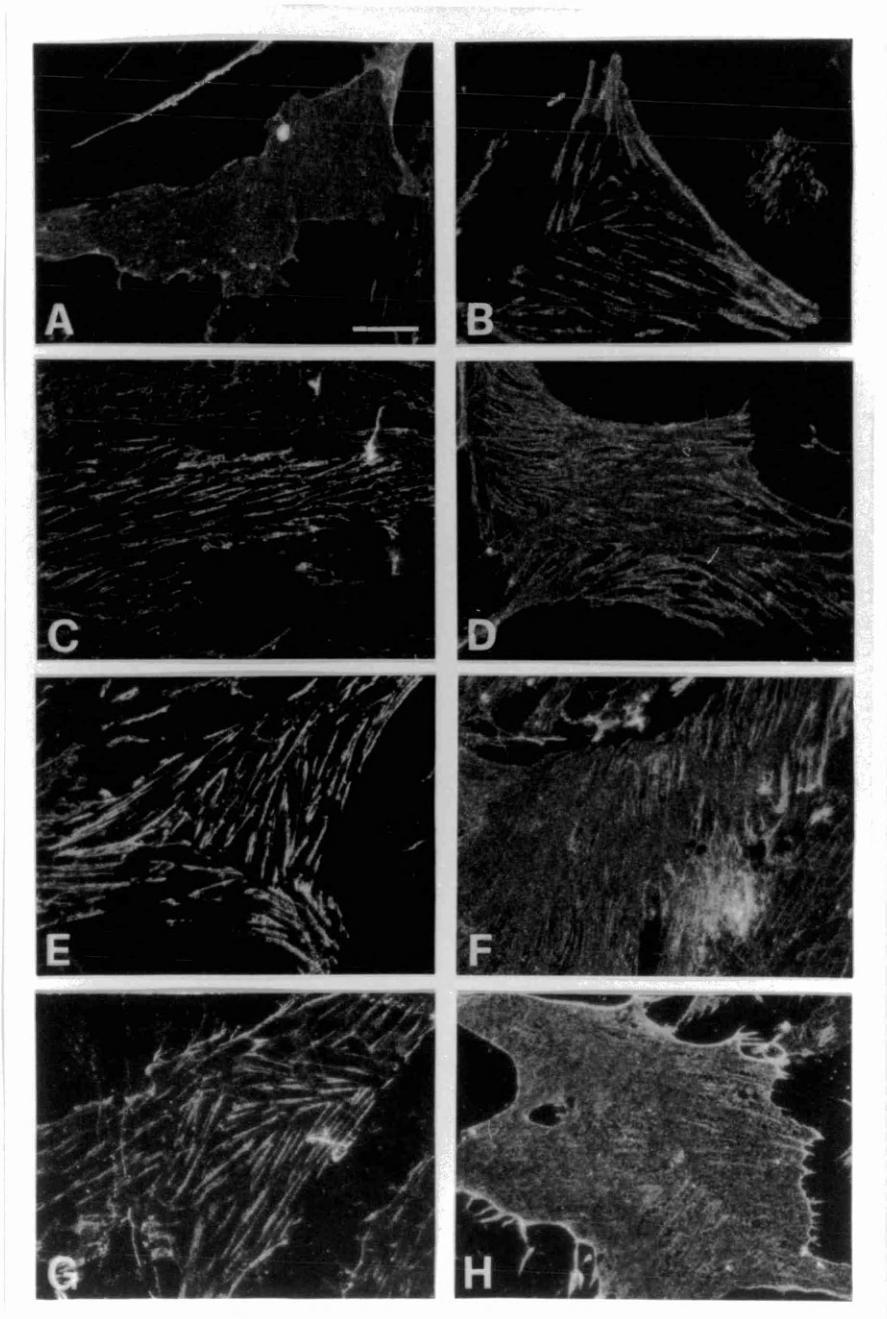


Figure 3.36. Function-regulating mAbs interfere with calcium-induced β1 redistribution

VPMs were incubated 15 min at 37°C in HCB in the absence (panel A), or presence (panel B) of the mAb TASC (20 μg/ml); in panels C and D, VPMs were incubated 15 min at 37°C in LCB, followed by 15 min at 37°C in HCB; in panels E and F, VPMs were treated at 37°C in HCB, followed by 15 min at 37°C in LCB. During all treatments, samples were incubated in the absence (panel C and E) or presence (panels D and F) of 200 μg/ml of the function-blocking mAb CSAT. In panels G and H, intact CEFs were incubated for 1 h at 37°C in extracellular buffer (see MATERIALS AND METHODS) in the presence of the activating mAb TASC (ascite, 1:30), or with 200 mg/ml of the function-blocking mAb CSAT, respectively. At the end of the incubation, cells were cooled on ice for preparation of VPMs. The samples were fixed and processed for immunofluorescence using the β1-cyto antibody, revealed by FITC-conjugated anti-rabbit IgG. bar = 10 μm.

experimental conditions.

TASC-dependent relocation of the receptors along ECM fibrils was also observed in intact CEFs (Fig. 3.36G). Moreover, incubation of CEFs with the function-blocking mAb CSAT induced diffusion of integrins from focal adhesions (Fig. 3.36H). These results point out that antibody-induced alteration of integrin distribution can occur both *in vivo* and *in vitro*.

3.4.4 β1 integrin redistribution on VPMs does not require the presence of stress fibers and the accumulation of focal adhesion components.

Integrin receptors colocalised with vinculin into focal adhesions of control, untreated VPMs (Fig. 3.37A and D, respectively). Interestingly, incubation for 15 min at 37°C either in HCB (Fig. 3.37B and E) or in LCB (Fig. 3.37C and F) led to loss of vinculin staining from VPMs (Fig. 3.37E and F). All the effects observed on integrin and vinculin distribution were negligible when VPMs were incubated at 0°C under the same experimental conditions (not shown).

To confirm this result, and to extend the analysis to other focal adhesion components, we performed immuno-blot analysis on lysates from control and experimentally-treated VPM preparations. Control samples solubilised immediately after preparation at 0°C (Fig. 3.38A, lanes 1 and 3), were compared to VPMs incubated 15 min at 37°C in LCB (Fig. 3.38A, lane 2), or HCB (Fig. 3.38A, lane 4), respectively. The results show that incubation at 37°C with either LCB or HCB did not affect the recovery of the integrin β1 subunit compared to the respective controls, as expected for a transmembrane protein. Actin was also largely retained on VPMs under all conditions analysed. In contrast, the recovery of several cytosolic components of the focal adhesions, such as tensin, talin, vinculin, and paxillin, was dramatically decreased after incubation at 37°C at either high or low [Ca^{2+}] (Fig. 3.38A, compare lanes 2 and 4 with lanes 1 and 3, respectively). These biochemical data were confirmed by immunofluorescence with antibodies specific for the different focal adhesion proteins (not shown).

Our data imply that low [Ca^{2+}]-induced relocation of diffuse receptors along ECM fibrils in VPMs pretreated with high [Ca^{2+}] (see Fig. 3.28E) did not require the accumulation of the analysed focal adhesion components on the cytoplasmic side of the membrane. Loss of vinculin and other focal adhesion components from VPMs was not

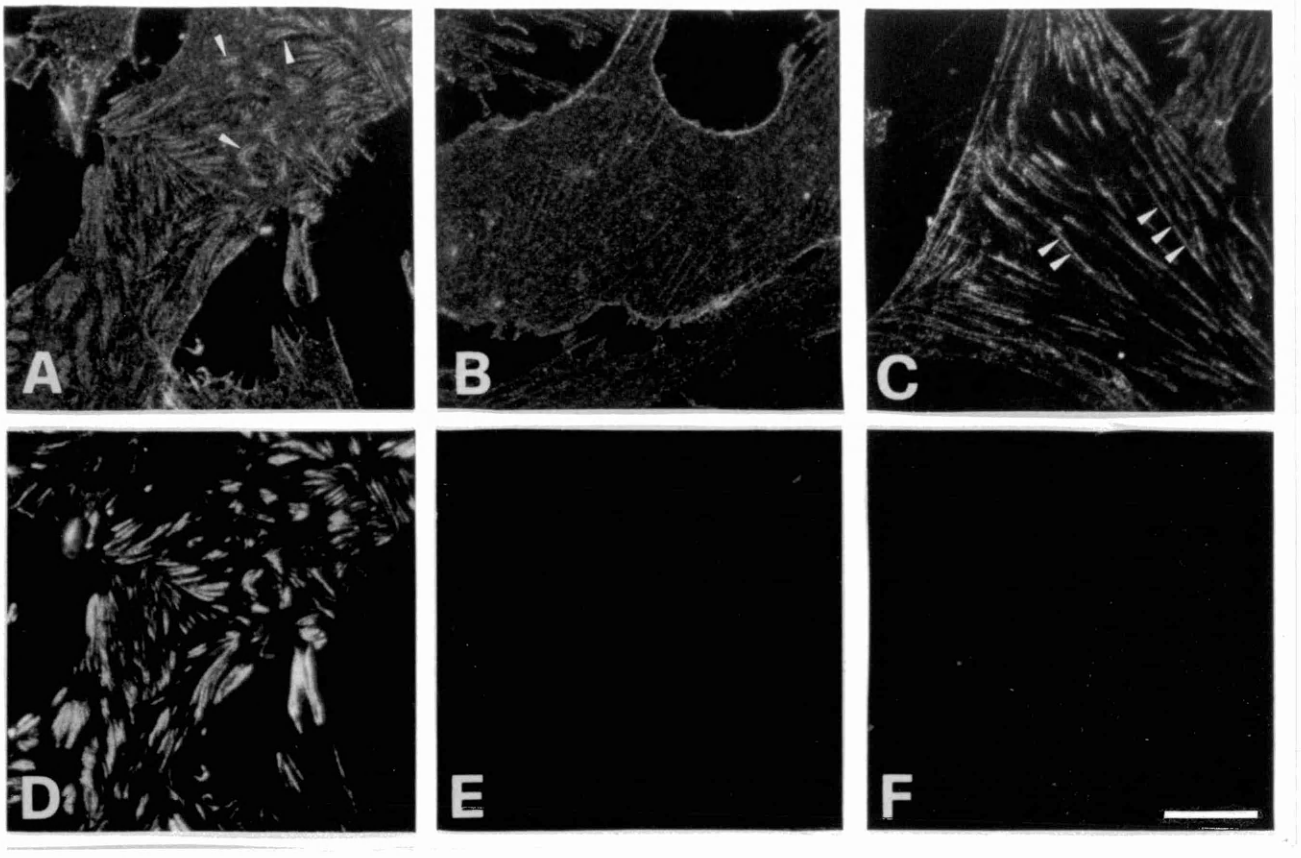


Figure 3.37. Loss of vinculin from focal adhesions during $[Ca^{2+}]$-induced integrin redistribution.

VPMs from adherent CEFs were fixed after preparation on ice (panels A and D), or after incubation for 15 min at 37°C in HCB (panels B and E) or in LCB (panels C and F). VPMs were then processed for immunofluorescence using the β1-cyto polyclonal antibody (panels A, B, and C), and the anti-vinculin mAb (panels D, E, and F). The same fields are shown in panels A and D, B and E, C and F. Arrowheads in panels A and C point to focal adhesions and fibrillary structures, respectively. Bar = 10 μm.

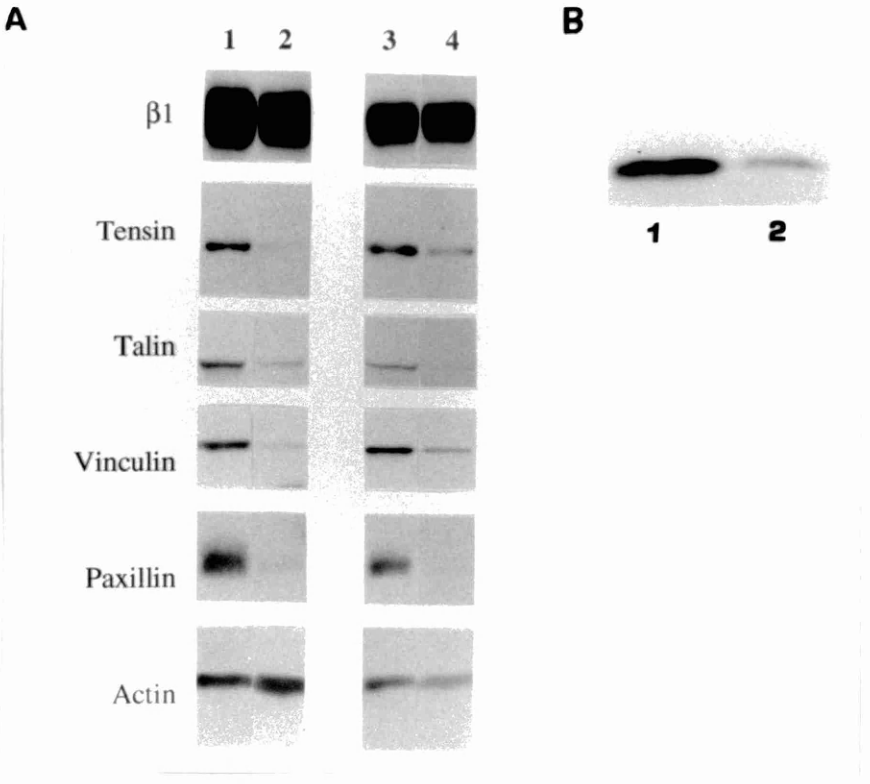


Figure 3.38. Loss of focal adhesion components from VPMs after incubation at 37°C, and actin depletion by gelsolin treatment.

Panel A: VPMs were extracted in SDS-PAGE buffer immediately after preparation on ice (lanes 1 and 3), or after incubation for 15 min at 37°C in LCB (lane 2), or in HCB (lane 4). Equal volumes of the lysates were run on a 5-13% polyacrylamide gradient gel, and processed for immuno-blotting using the following primary antibodies: β1-cyto (β1), anti-tensin, anti-talin, anti-vinculin, anti-paxillin, and anti-actin. Primary polyclonal and monoclonal antibodies were revealed by ^{125}I-protein A or ^{125}I-sheep anti-mouse IgG, respectively. Loss of focal adhesion components was evident after incubation of VPMs at 37°C in the presence of either low (lane 2) or high (lane 4) [Ca^{2+}]; β1 integrins and actin were not significantly affected by either treatment.
Panel B: VPMs from adherent fibroblasts were incubated for 3 min at 0°C in HCB (lane 1), or HCB containing 4 μM gelsolin, before extraction in SDS-PAGE buffer. Equal volumes of lysates were loaded on a 7% polyacrylamide gel and processed for immunoblotting with anti-actin mAb. The primary antibody was revealed by ^{125}I-sheep anti-mouse IgG.

simply due to warming of the samples at 37°C. In fact, under similar experimental conditions, loss of focal adhesion proteins could be prevented by incubation of VPMs at 37°C in buffers of lower ionic strength, or with more acidic pH (below pH 6, not shown).

To investigate the role of actin on [Ca^{2+}]-mediated β1 integrins redistribution *in vitro*, we selectively disassembled the stress fibers by using the actin capping and severing protein gelsolin. Incubation of VPMs for 3 min at 0°C in the presence of 4 μM recombinant gelsolin was sufficient to induce loss of more than 90% of actin present on VPMs, as revealed by immuno-blotting (Fig. 3.38B). As confirmed by F-actin staining with fluorescent phalloidin (Fig. 3.39D), treatment with gelsolin was able to disassemble most, if not all stress fibers. Some F-actin could still be detected only in areas at the border of VPMs, where residues of the dorsal plasma membrane were probably preventing efficient access of gelsolin (Fig. 3.39E and F, asterisks). This residual actin could justify the presence of approximately 10% actin in gelsolin-treated VPMs, as detected by immuno-blotting (Fig. 3.38B). In VPMs maintained at 0°C, β1 integrins could be detected in focal adhesions even in the absence of detectable stress fibers (compare Fig. 3.39C and D, with 3.39A and B, respectively). Furthermore, high [Ca^{2+}]-induced diffusion of the receptors (Fig. 3.39E), and subsequent low [Ca^{2+}]-induced concentration along ECM fibrils (Fig. 3.39G) were not affected by lack of stress fibers (Fig. 3.39F and H, respectively). These results show that stress fibers are not required for the redistribution of β1 integrins under cell-free conditions. Moreover, removal of stress fibers from VPMs does not affect maintenance of β1 integrins into focal adhesions at 0°C.

3.4.5 The distribution of β1 integrins lacking the cytoplasmic domain is affected by a mAb modulating integrin function, but not by [Ca^{2+}]

To study whether interactions between integrins and intracellular factors may play a role in receptor redistribution, we transfected CEFs with the β1TR construct corresponding to the human β1 integrin lacking the entire cytoplasmic domain. The distribution of β1TR on VPMs was analysed by immunofluorescence using the mAb TS2/16, specific for the human β1 subunit. As expected, we found that in contrast to the endogenous β1 receptor (Fig. 3.40A), β1TR was not clustered in focal adhesions, but

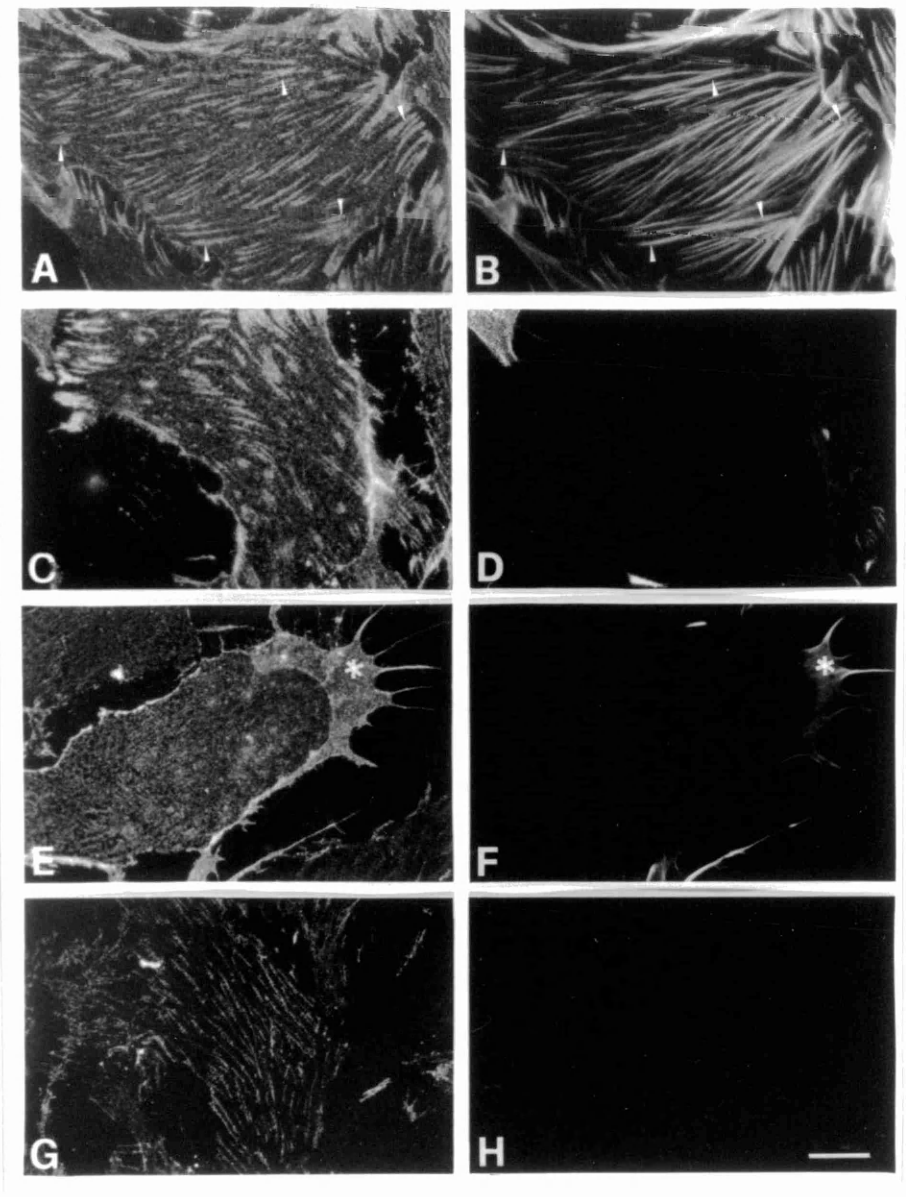


Figure 3.39. Maintenance of β1 integrins in focal adhesions, and their calcium-dependent redistribution are not affected by gelsolin-induced stress fibers disassembly under cell-free conditions.

VPMs from adherent fibroblasts were fixed immediately after preparation on ice (panels A and B), or were incubated 3 min at 0°C with 4 μM gelsolin to disrupt F-actin (panels C-H). After the treatment with gelsolin, VPMs were either fixed (panels C and D), or incubated 15 min at 37°C in HCB (panels E-H). VPMs were then fixed (panels E and F), or incubated for further 15 min at 37°C in LCB (panels G and H). After fixation, all samples were processed for immunofluorescence with the β1-cyto antibody, and TRITC-conjugated anti-rabbit IgG (panels A, C, E and G). FITC-phalloidin (panels B, D, F, and H) was used to stain F-actin. The same fields are shown in panels A and B, C and D, E and F, G and H. Arrowheads in panels A and B show colocalisation of integrins in focal adhesions A, with the tip of actin stress fibers (panel B). Asterisks in panels E and F show areas at the periphery of the VPM where a portion of the dorsal plasma membrane is present, and some F-actin is still visible. Bar = 10 μm.

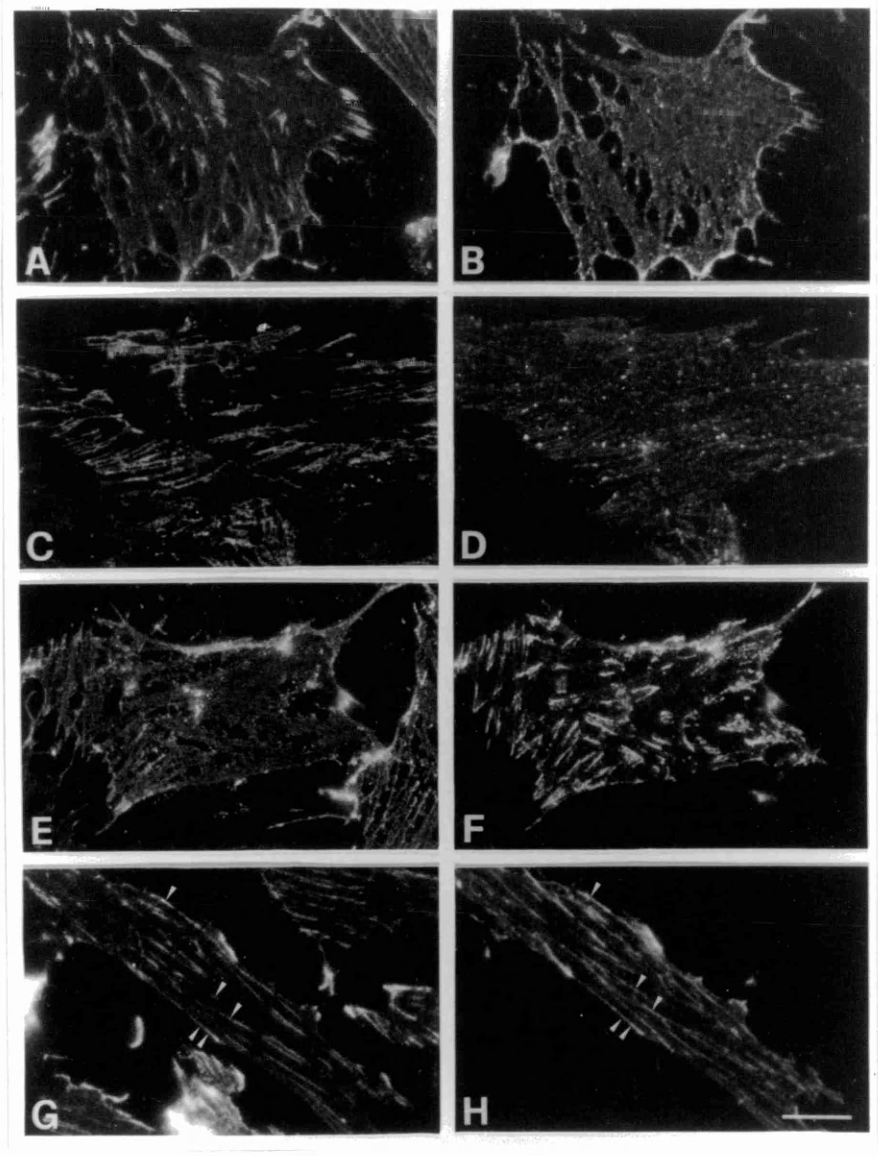


Figure 3.40. Low [Ca^{2+}] does not affect the localisation of integrin β1 receptors lacking the cytoplasmic domain.

CEFs were transiently transfected to express the truncated β1TR construct corresponding to the human integrin β1 subunit lacking the cytoplasmic domain. After 20 h culture, VPMs were prepared at 0°C and immediately fixed (panels A and B), or incubated for 15 min at 37°C in LCB (panels C and D), or in HCB containing 40 μg/ml of the activating mAb TS2/16 specific for the human β1 subunit (panels E and F). In panels G and H, intact transfected CEFs were incubated for 1 h at 37°C with 40 μg/ml of the activating mAb TS2/16 specific for the human β1 subunit, in a buffer containing 20 mM Hepes, 2.7 mM KCl, 137 mM NaCl, 5.6 mM glucose, 0.2 mMEGTA, 1 mM $MgCl_2$, pH7. After fixation, the samples were incubated with the β1-cyto antibody and FITC-conjugated anti-rabbit IgG to detect the endogenous chicken β1 subunit (panels A, C, E, and G), while the truncated human β1 subunit was detected with the anti-human β1 mAb TS2/16, revealed by TRITC-conjugated anti-mouse IgG (panels B, D, F, and H). The same fields are shown in panels A and B, C and D, E and F, G and H. Bars = 10 μm.

showed a diffuse staining on VPMs fixed immediately after preparation at 0°C (Fig. 3.40B). After incubation in LCB, β1TR was found predominantly diffuse, without a clear colocalisation with the endogenous receptor (Fig. 3.40C and D). As expected, β1TR had a diffuse localisation after incubation of VPMs in HCB (not shown).

In contrast, β1TR did clearly concentrate along ECM fibrils if VPMs were incubated 15 min at 37°C in the presence of the human-specific stimulatory mAb TS2/16. This effect was observed either at high [Ca^{2+}] (Fig. 3.40F), with the endogenous receptor diffuse on the membrane (Fig. 3.40E), or at low [Ca^{2+}], with the endogenous β1 receptors colocalising with β1TR in ECM fibrils (not shown). As observed for the endogenous wild type receptors in CEFs treated with the chicken-specific activating mAb TASC (Fig. 3.36G), incubation of intact CEFs with the human-specific activating antibody TS2/16 induced localisation of part of β1TR along ECM fibrils (Fig. 3.40H). Under these conditions, transfected β1TR colocalised with the endogenous receptor, concentrated into focal adhesions (Fig. 3.40G).

These results show that the mechanism of low [Ca^{2+}]-mediated β1 integrin redistribution along fibrils *in vitro* requires the cytoplasmic domain of the receptor, while receptors lacking the cytoplasmic domain retain the ability to redistribute *in vivo* and *in vitro*, upon activation by stimulatory antibodies.

3.5 RECONSTITUTION OF A RECEPTOR COMPLEX AND RECRUITMENT OF EXOGENOUS ACTIN AT FOCAL ADHESION SITES ON VPMs

By using VPMs as a "cell-free" system, we have demonstrated that it is possible to modulate integrin function and distribution on the membrane, by changing cations concentration in the buffer. Moreover, this "cell-free" system, has allowed the dissection of focal adhesion architecture, demonstrating an uncoupling of the receptor from the connected cytoskeleton. These results indicated that this may be a suitable model for the reconstitution of some of the molecular interactions required for the assembly of focal adhesions. In our system we can test the binding of purified components to VPMs in which β1 receptors affinity for the ligand can be modulated. For this purpose, we have used purified α-actinin, an actin-binding protein known to interact *in vitro* with the cytoplasmic tail of integrins (Otey et al., 1990). The results obtained showed that purified α-actinin colocalises and redistributes with β1 receptors, implicating binding of α-actinin to the receptors.

The relationships between focal adhesions and actin assembly is not well characterised. To start investigating this problem, we have used our "cell-free" system and found a special property of focal adhesion complexes in recruiting exogenous actin under conditions in which new actin polymerisation is inhibited.

3.5.1 Reconstitution of α-actinin binding to F-actin-depleted VPMs: [Ca^{2+}]-mediated redistribution under cell-free conditions

We have utilised purified α-actinin in binding experiments to VPMs, with the aim of reconstituting some of the molecular interactions which are believed to be important for focal adhesion assembly. This protein is known to bind both integrin and F-actin. In contrast to most of the focal adhesion proteins examined (see Fig. 3.38), some α-actinin remained along stress fibers after 15 min incubation at 37°C in either high or low [Ca^{2+}] buffers (Fig. 3.41B and D, respectively). Incubation of VPMs for 10 min at 37°C with purified α-actinin in high [Ca^{2+}] buffer led to the accumulation of the exogenous protein (Fig. 3.41H) predominantly along stress fibers (Fig. 3.41G). Binding of exogenous α-actinin was evident (Fig. 3.41F and H), when compared with the endogenous α-actinin remaining after incubation at 37°C in the absence of the exogenous protein (Fig. 3.41B and D). In both cases, the pattern of distribution of α-actinin was largely different from that of integrins (compare Fig. 3.41B with A, and F with E).

To test whether binding of α-actinin to β1 integrins could be reconstituted in this system, we repeated the experiments on VPMs depleted of F-actin (Fig. 3.39D). After incubation with gelsolin at 37°C at high [Ca^{2+}], no endogenous α-actinin could be detected on the membranes (Fig. 3.42E). Incubation of these membranes for 10 min at 37°C in the presence of purified α-actinin at high [Ca^{2+}] resulted in a strong, diffuse signal on the membranes (Fig. 3.42F), which was similar to that observed for β1 integrins (Fig. 3.42C).

Treatment of VPMs at 37°C with gelsolin at low [Ca^{2+}] resulted also in complete removal of the endogenous α-actinin (Fig. 3.42J), and further incubation with purified α-actinin at low [Ca^{2+}] resulted in a fibrillar staining (Fig. 3.42K), which overlapped with the staining for the β1 receptors (Fig. 3.42H). Moreover, binding of exogenous α-actinin to gelsolin-treated VPMs at high [Ca^{2+}], followed by incubation at 37°C at low [Ca^{2+}] in absence of the exogenous protein, resulted in the redistribution of both pre-bound α-actinin and integrins along ECM fibrils (Fig. 3.42I and L).

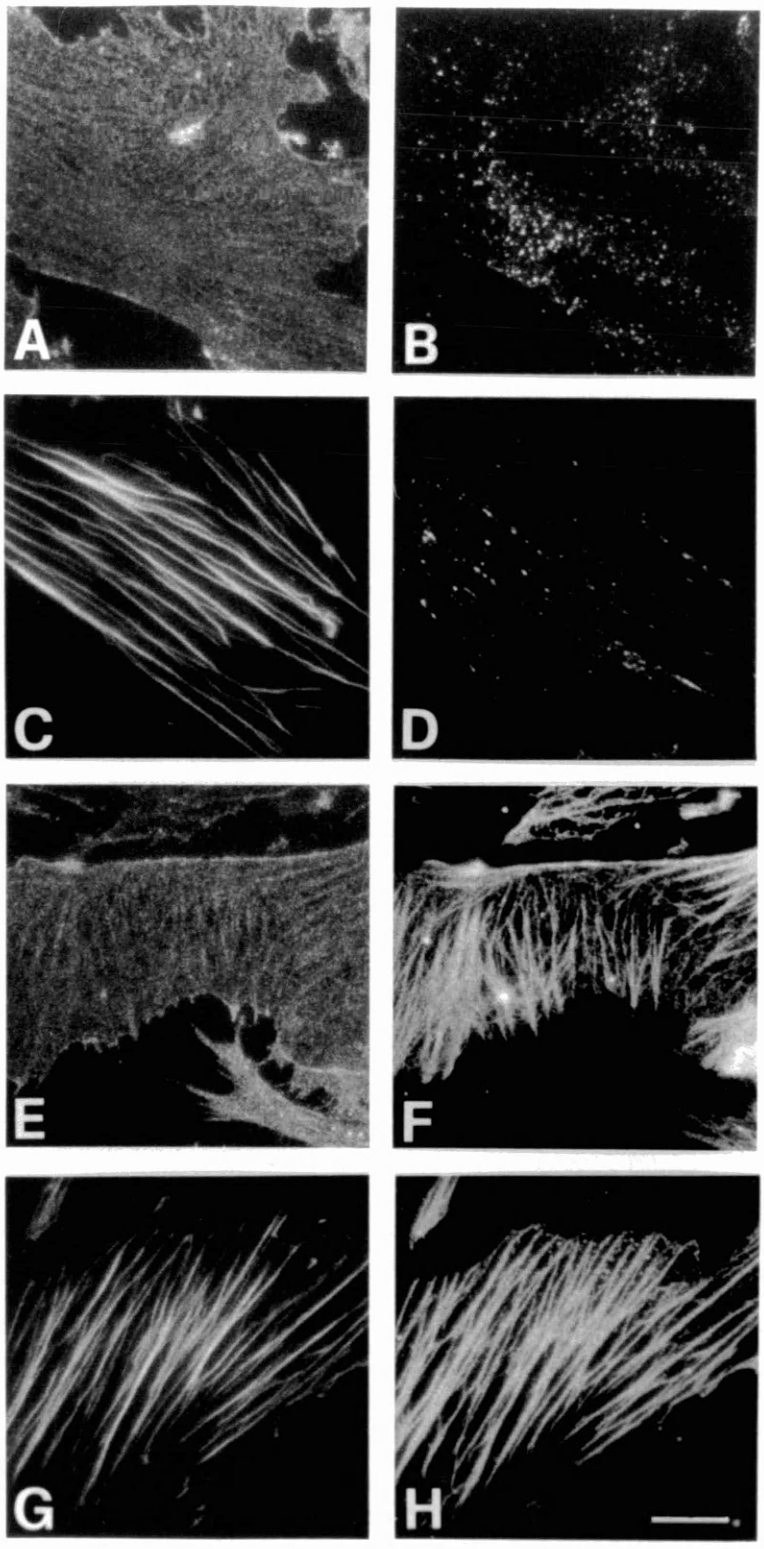


Figure 3.41. Reconstitution of the binding of α-actinin to VPMs in the presence of actin stress fibers.

After a first incubation for 10 min in low ionic strength, high [Ca^{2+}]-containing buffer, VPMs were incubated for further 10 min in the same buffer in the absence (panels A-D), or presence (panels E-H) of purified α-actinin, as described in chapter 2. After fixation, F-actin was detected with FITC-phalloidin (panels C and G), β1 integrins were detected with the β1-cyto antibody (panels A and E), and α-actinin was detected with a specific mAb (panels B, D, F, and H). Same fields are shown in panels A and B, C and D, E and F, G and H. Bar = 10 μm.

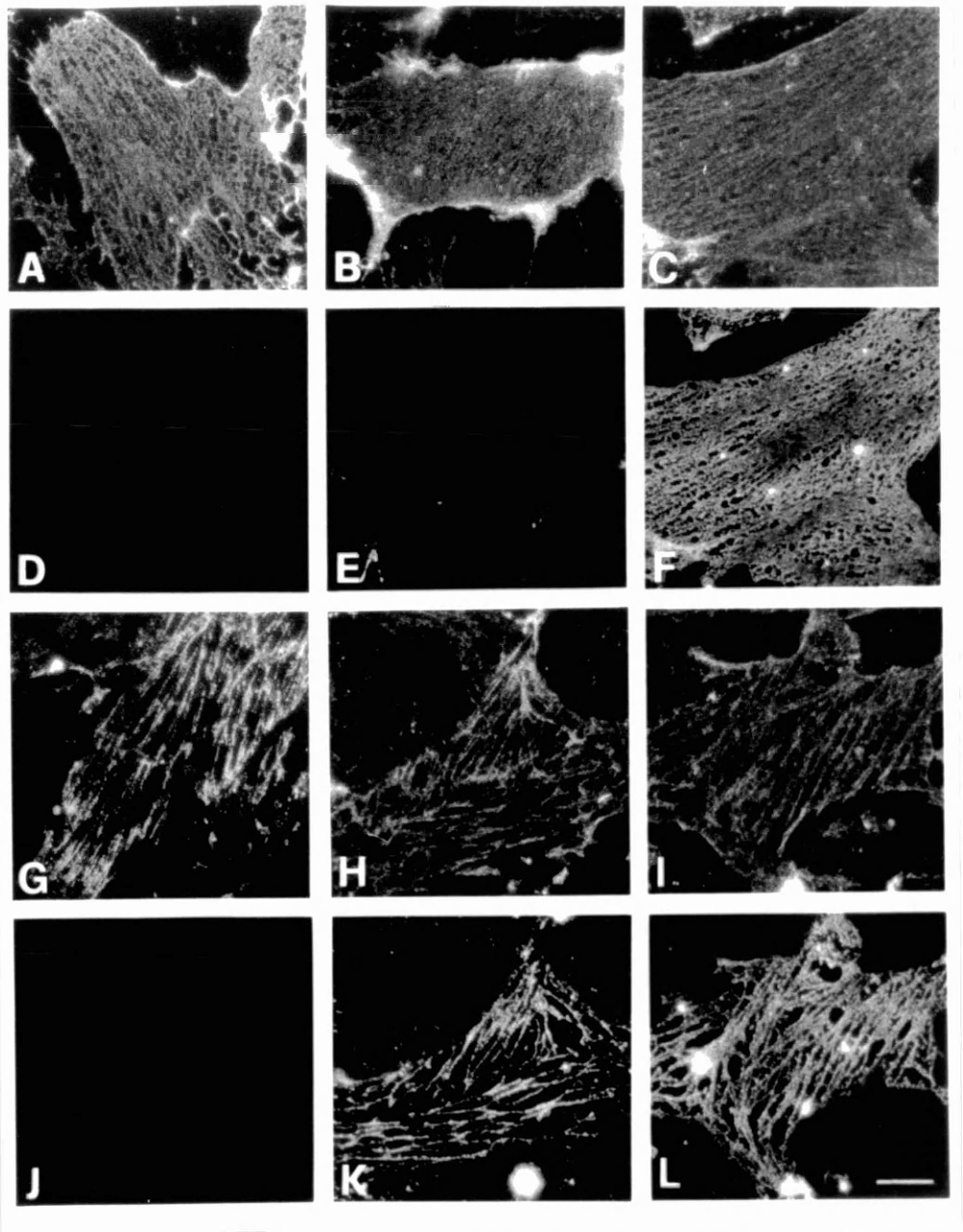


Figure 3.42. Reconstitution of the binding of α-actinin to VPMs in the absence of F-actin.

VPMs were incubated for 10 min in low ionic strength buffer containing 4 μM gelsolin, at high [Ca^{2+}] (panels A-F, I, L), or low [Ca^{2+}] (panels G, H, J, K). The samples were further incubated at 37°C in a buffer with high [Ca^{2+}] (panels A-F, I, L), or low [Ca^{2+}] (panels G, H, J, K). In panels C, F, H, I, K, and L, the second incubation was in the presence of purified α-actinin, as detailed in the Methods. Membranes were then washed and fixed for immunofluorescence. For low [Ca^{2+}]-induced α-actinin redistribution (panels I and L), after the second incubation at 37°C for 10 min with α-actinin in high [Ca^{2+}]-containing buffer, the samples were immediately transferred to low [Ca^{2+}]-containing buffer and incubated for 10 min at 37°C, to induce integrin redistribution along ECM fibrils before fixation. Panels A-C and G-I, integrin distribution, by using the β1-cyto polyclonal antibody; panels E, F, and J-L, localisation of α-actinin with the specific mAb; Panel D, F-actin localisation with TRITC-phalloidin. Same fields are shown in panels A and D, B and E, C and F, G and J, H and K, I and L. Bar = 10μm.

These data provide evidence for the reconstitution of the binding of a focal adhesion component to β1 receptors in VPMs. Moreover, the $[Ca^{2+}]$-induced redistribution of partially reconstituted adhesive complexes under cell-free conditions, illustrates the possibility to use this system for further reconstitution studies.

3.5.2 Recruitment of exogenous actin to focal adhesion sites on VPMs

To start investigating the relationships between focal adhesions and actin assembly in our cell-free system, we performed a number of experiments utilising Rh-actin. We found that incubation of freshly prepared VPMs with 1 μM Rh-actin for 2 min at 37°C, a condition which preserved the localisation of vinculin into focal adhesions (Fig. 3.43A), resulted in the accumulation of the exogenous actin in fibrillar structures on the membranes (Fig. 3.43D). Under these conditions, exogenous actin colocalised with stress fibers (compare Fig. 3.43B and E). When Rh-actin was added to VPMs in the presence of cytochalasin D and DNase I (which inhibit polymerisation from the barbed and pointed end of actin, respectively), exogenous actin accumulated specifically to focal adhesion sites (Fig. 3.43C and F), while binding along stress fibers was not detected. The same result was obtained by incubation with cytochalasin D only (not shown). Since the short incubation with cytochalasin D and DNase I did not evidently affect endogenous stress fibers (not shown), this result indicates that accumulation along stress fibers requires new actin polymerisation, and is somehow different from binding of exogenous actin to focal contacts.

Exogenous actin accumulated along stress fibers on VPMs pre-incubated for 15 min at 37°C at low $[Ca^{2+}]$ (Fig. 3.43J), and had a distribution distinguishable from the fibrillar pattern observed for β1 (Fig. 3.43G). Cytochalasin D and DNase I completely prevented actin recruitment on VPMs under these conditions (Fig. 3.43K). Since incubation for 15 min at 37°C in low $[Ca^{2+}]$ resulted in the absence of detectable levels of several focal adhesion proteins on VPMs (e.g., vinculin: see Fig. 3.37F), these data indicate that focal adhesion complexes are required to specifically recruit actin under conditions in which new actin polymerisation is inhibited.

With the aim of starting to analyse the minimal requirements for the recruitment of actin at adhesive sites, we tried to reconstitute Rh-actin binding to partially reconstituted adhesive complexes in the presence of actin polymerisation inhibitors. VPMs containing exogenous α-actinin were prepared as described in the previous paragraph (see Fig.

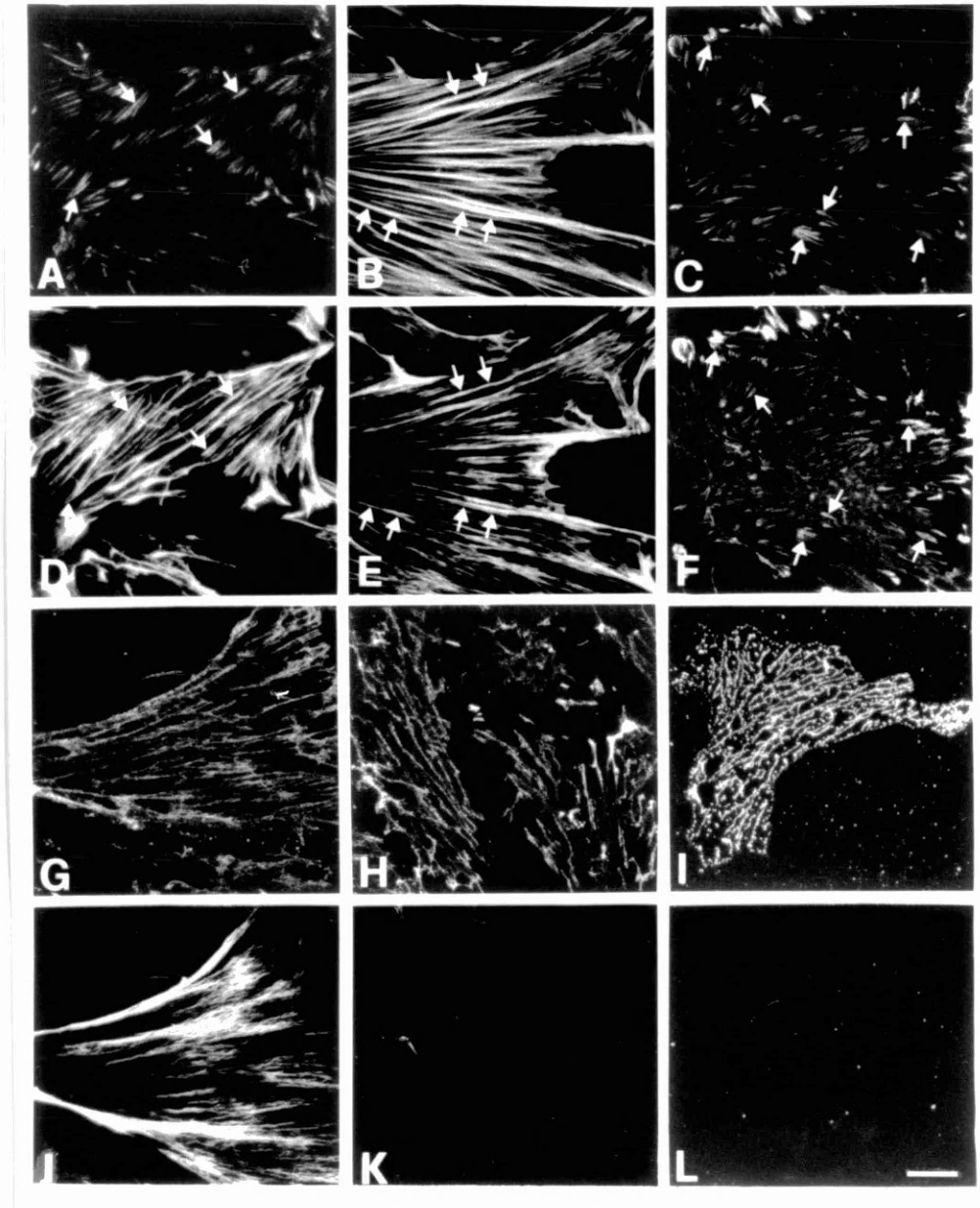


Figure 3.43. Accumulation of exogenous actin at focal adhesion sites is not affected by inhibitors of actin polymerisation.

VPMs were incubated for 2 min at 37°C in LCB with 1 μM Rh-actin either immediately after preparation (panels A-F), or after 15 min incubation at 37°C in LCB (panels G, H, J, and K). In panels I and L, VPMs were incubated with exogenous α-actinin (as described in the legend of Fig. 3.41L), before incubation for 2 min at 37°C with Rh-actin in LCB. In panels C, F, H, I, K, and L, 100 nM cytochalasin D and 100 μg/ml DNase I were present during the incubation with Rh-actin. After fixation, the samples were processed for immunofluorescence with the anti-vinculin mAb (panels A and C), the β1-cyto polyclonal antibody (panels G and H), or the anti-α-actinin polyclonal antibody (panel I). In panel B, F-actin was detected with FITC-phalloidin, while in panels D, E, F, J, K, and L, bound Rh-actin is shown. Arrows in panels A and D, C and F, point to sites where Rh-actin colocalises with vinculin in focal adhesions, while arrows in panels B and E indicate colocalisation of Rh-actin with stress fibers. The same field is shown in panels A and D, B and E, C and F, G and J, H and K, I and L. Bar = 10 μm

3.42L), and incubated for 2 min at 37°C with 1 μM Rh-actin in the presence of cytochalasin D and DNase I. Under these conditions, detectable levels of Rh-actin could not be observed on the membranes. This result suggests that complexes between active β1 integrins and α-actinin are not sufficient to recruit exogenous actin to sites of interaction between the cell and the ECM.

- Chapter 4 -

DISCUSSION

4.1 MORPHOLOGICAL CHARACTERISATION OF VPMs: VPMs retain well preserved focal adhesions and stress fibers

In this study, I have used a modification of the lysis-squirting technique (Avnur and Geiger, 1981; Nermut *et al.*, 1991), for the preparation of detergent-free ventral plasma membranes (VPMs) obtained from adherent chicken embryo fibroblasts (CEFs). By a combination of different morphological (indirect immunofluorescence, phase contrast, interference reflection and scanning force microscopy) and biochemical techniques, I have shown that known focal adhesion components and the connected actin cytoskeleton are retained on the membrane after the hypotonic treatment and the mechanical cell disruption for VPMs preparation, suggesting that relatively strong interactions are involved in the maintenance of these structures.

An advantage of using VPM preparations is the possibility of studying the distribution of antigens on the ventral surface of the cell and on the connected cytoskeleton, without interference coming from the signal inside the cell or from the dorsal portion of the membrane. We have already successfully utilised VPMs for the study of the differential subcellular distribution of two cytoplasmic variants of the $\alpha 6\beta 1$ integrin laminin receptor (Cattelino *et al.*, 1995). Here I reported the distribution of the small GTP-binding protein Rac 1 along actin stress fibers and in focal adhesions in VPM preparations (Fig. 3.5), while the same protein showed a typical late endosomal, lysosomal pattern in intact cells, according to pervious reports in other cell types (Ridley *et al.*, 1992; Robertson *et al.*, 1995). Although it is well known that Rho family GTP-binding proteins, including Rac, are implicated in the dynamic organisation of the actin cytoskeleton (Nobes and Hall, 1995), to my knowledge, this is the first indication of the localisation of a Rho GTPase at the actin cytoskeleton. In line with this result, we have recently demonstrated that the overexpressed wild type Rac 1 has the same localisation of the endogenous protein in VPMs prepared from CEFs, and that the overexpression of this GTPase induces disassembly of stress fibers, and production of long, highly

branched actin-rich protrusions, with consequent dramatic changes in cell morphology (Albertinazzi *et al.*, in press).

Ezrin is a component of the ERM (Ezrin/Radixin/Moesin) family of proteins, specifically localised to microvilli in a number of polarised epithelia (Bretscher, 1983; Hanzel *et al.*, 1991; Berryman *et al.*, 1993;, Franck *et al.*, 1993;, Winckler *et al.*, 1994; Amieva and Furthmayr, 1995). The use of VPMs has allowed us to visualise the localisation of this protein at focal adhesion sites (Fig. 3.6). Interestingly, radixin, another member of the family, has been also localised to focal adhesions using a similar approach (Sato *et al.*, 1992). This finding, together with the finding that ERM family proteins can reconstitute actin polymerisation and focal contact formation in response to activation of Rho and Rac in serum-starved 3T3 fibroblasts permeabilised with digitonin (McKay *et al.* 1997), strongly indicates a role of these proteins in the regulation of focal adhesion sites, and of the connected actin cytoskeleton.

Scanning force microscopy was useful for a general, high resoluting view of the topography of VPMs. While indirect immunofluorescence enhances the contrast of selectively revealed antigenic structures, their organisation with respect to adjacent, unlabeled components cannot be determined. In scanning force microscopy, the height of any membrane component that is significantly different from its surroundings is contrasted and quantitated. In topographs of VPMs different structures could be revealed: 100 nm-diameter particles, probably corresponding to early endosomes (Fig. 3.7); holes in the lipid bilayer (Figs 3.7 and 3.8); unlabeled filaments extending beyond the perimeter of the membrane, probably corresponding to extracellular matrix fibers (Fig. 3.9); stress fibers and focal contacts. Focal contacts were always visible in scanning force micrographs unless overlaid by bulky stress fibers. On ventral plasma membranes prepared from cells cultured for 1.5-3 hours, large focal contacts were common at the periphery. These focal adhesions exhibited fine fibrillar structures probably related to actin networks associated with stress fiber terminals (Fig. 3.10). Such fine fibrillar structures could not be revealed in fluorescence images of the same membranes labelled for actin and vinculin.

All together these results evaluate the system for further studies on focal adhesion composition. With the aim of obtaining new informations about focal adhesion composition and regulation, we have injected VPMs preparations into mice for the production of monoclonal antibodies. The injection of a miscellaneous material has been a successful approach in a number of other systems for the production of monoclonal antibodies, and for the identification of new polypeptides. In our case, the approach used

was successful for the production of antibodies against components of the extracellular matrix and of the actin cytoskeleton, including some actin-binding proteins, which need to be further characterised. Unfortunately, despite the presence of well-preserved focal adhesions in VPM preparations, we were unable to obtain antibodies against either known or new focal adhesion components. This could be due to the relatively scarce amount, exposure, or antigenity of single focal adhesion components as compared to other structures present in VPM preparations.

4.2 BIOCHEMICAL CHARACTERISATION OF VPMs: tyrosine-phosphorylated paxillin is preferentially localised at focal adhesion sites

The results presented in 3.2 show the specific accumulation of several tyrosine-phosphorylated polypeptides at focal adhesions in VPMs, and the preferential localisation of the tyrosine-phosphorylated form of focal adhesion components, such as paxillin, at focal adhesion sites. These results indicate that tyrosine-phosphorylated proteins are constitutive components of the adhesion sites of CEFs, where they may play an important role in focal adhesion formation and maintenance.

Ligand-dependent integrin clustering has been shown to be sufficient to induce the aggregation and colocalisation of several focal adhesion molecules at the adhesion sites (Miyamoto *et al.*, 1995), although the mechanisms involved are poorly understood. Tyrosine phosphorylation is thought to play an important role in integrin signalling and integrin-mediated cytoskeletal reorganisation, but no direct functional evidence on the role of tyrosine phosphorylation in cell adhesion to the extracellular matrix is available yet. Elevated phosphotyrosine has been detected in focal adhesions of transformed (Comoglio *et al.*, 1984) and non-transformed cells (Maher *et al.*, 1985). Since the levels of tyrosine phosphorylation of focal adhesion components are generally low, it has been recently proposed that the pools of proteins in focal adhesions may contain much higher levels of phosphate compared to the total cellular pools (Pavalko and Otey, 1994), but direct evidence for this hypothesis has been missing until now. To be able to investigate this issue, we have used the VPM preparation. The results obtained from the biochemical analysis show that VPMs are highly enriched in tyrosine-phosphorylated proteins when compared to whole cell lysates. The fact that hypotonic treatment and mechanical

disruption, while releasing most of the cytosolic proteins, did not disassemble focal adhesions, suggests that relatively strong interactions are involved in the maintenance of these structures, which could be disrupted by detergent extraction. In fact, although a detergent-insoluble pellet remained after extraction of VPMs with Triton X-100, we found that most of the tyrosine-phosphorylated proteins and of other focal adhesion components were recovered in the detergent-soluble fraction, while part of the actin cytoskeleton remained insoluble (Fig. 3.11). Tyrosine-phosphorylated components were analysed in CEFs that had spread on the substrate for 18 h before preparation of VPMs, suggesting that high levels of phosphorylation are preserved after the initial formation of focal adhesions, and may be required for maintenance and/or continuous reorganisation of the adhesive sites in these cells. Furthermore, the localisation studies presented here indicate that tyrosine-phosphorylated proteins recovered in VPM preparations are concentrated in the focal adhesions.

Well-characterised focal adhesion components, such as vinculin, talin and paxillin, can be clearly localised to focal adhesion in VPMs by immunofluorescence, but they show only a weak or no enrichment in VPMs compared to whole cell lysates. This finding is an indication of the existence of large cytosolic pools, which are not associated to the adhesion sites. This is evident for paxillin, where the diffuse intracellular staining observed in fixed, permeabilised cells disappears in VPMs, where only focal adhesion staining for paxillin can be observed.

One explanation for these findings comes from the analysis of the distribution of tyrosine-phosphorylated paxillin. As previously reported (Turner *et al.*, 1990; Turner, 1991; Turner *et al.*, 1993; Glenney and Zokas, 1989), we found that tyrosine-phosphorylated paxillin represents only a minor fraction of the polypeptide found in whole cell lysates. On the other hand, our results indicate that the level of tyrosine-phosphorylated paxillin detected in VPM lysates is close to 100%. Therefore, although the total paxillin polypeptide is not enriched in VPMs, tyrosine-phosphorylated paxillin is highly enriched in these preparations (see scheme of Fig. 4.1). This finding, together with the specific localisation of paxillin in focal adhesions of VPMs by immunofluorescence, indicates the preferential localisation of a minor pool of paxillin with a high level of tyrosine-phosphorylation at focal adhesion sites.

We were not able to determine the level of tyrosine phosphorylation of FAK in the VPMs, since we could not detect a band above background levels in FAK immunoprecipitates blotted with anti-FAK antibodies under our experimental conditions (data not shown). On the other hand, quantitation of the specific 120KD tyrosine-

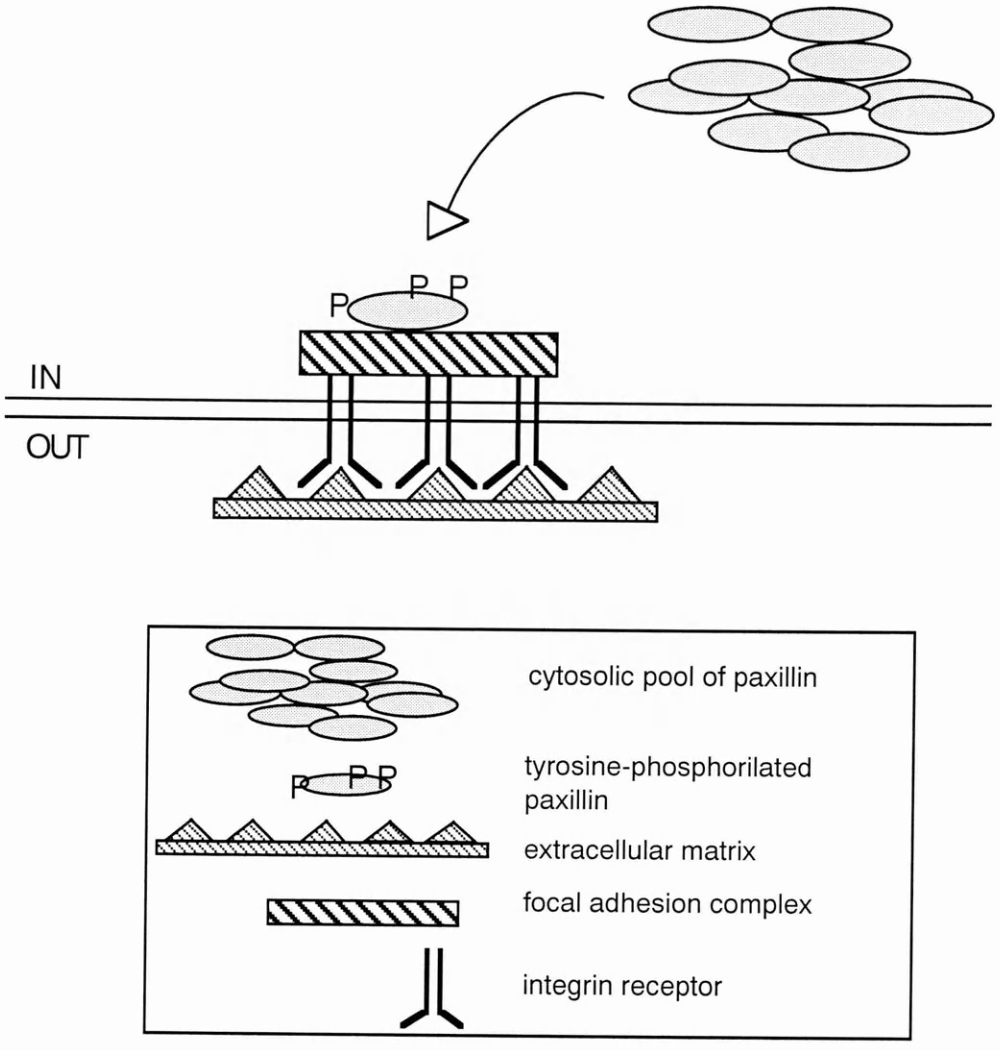


Figure 4.1. Schema of the accumulation of tyrosine-phosphorylated paxillin at sites of focal adhesion.

Tyrosine-phosphorylated paxillin represents only the 10% of the pool of paxillin present in the cell, and it is selectively accumulated at sites of focal adhesions. At these sites, integrin receptors are clustered and bound to the extracellular ligand from one side, and to intracellular focal adhesion components from the other side. ~~phosphorylated band immunoprecipitated with FAK antibodies showed an enrichment of the tyrosine-phosphorylated form of FAK in VPM lysates.~~

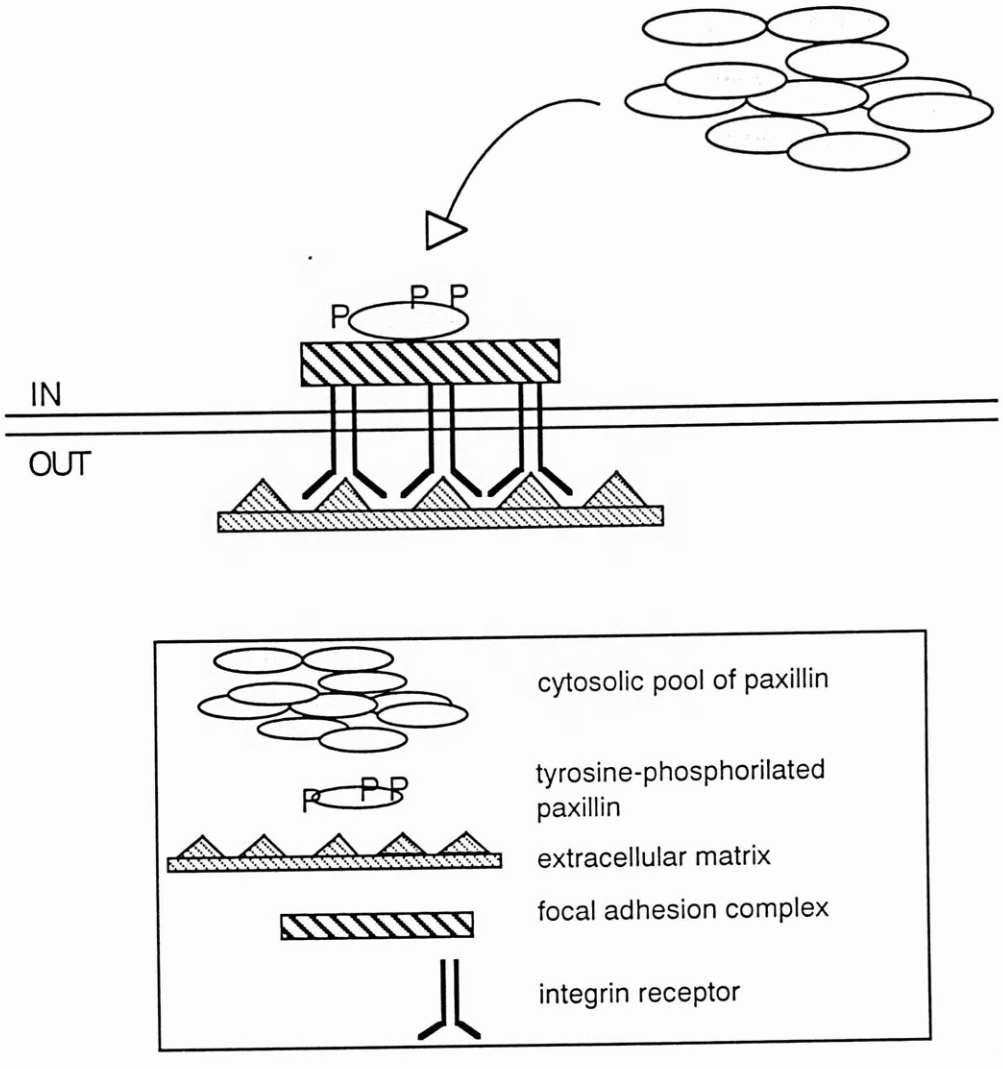
FAK and paxillin are two of the best characterised focal adhesion components, and they are tyrosine-phosphorylated during cell adhesion to the extracellular matrix (Burridge *et al.*, 1992; Kornberg *et al.*, 1992). While FAK may regulate the function of focal adhesion components by phosphorylation, paxillin is believed to play a role in the assembly of the focal adhesions, by forming complexes with several components of the adhesive sites, as shown by *in vitro* and *in vivo* studies (Birge *et al.*, 1993; Hildebrand *et al.*, 1995; Sabe *et al.*, 1994; Turner and Miller, 1994; Weng *et al.*, 1993). Although *in vitro* studies have shown that the SH2 domains of Crk, Csk, Src, and Fyn can recognise tyrosine-phosphorylated paxillin (Schaller and Parsons, 1995), the role of paxillin tyrosine-phosphorylation *in vivo* remains to be established. The use of mutants of a major paxillin phosphorylation site (tyrosine 118) *in vivo* (Schaller and Parsons, 1995) indicates that phosphorylation of this site is not required for recruitment to focal adhesions (Bellis *et al.*, 1995), although a number of other phosphorylation sites exist on paxillin that may perform this function. Alternatively, paxillin may become phosphorylated once recruited to the adhesion site by a different mechanism. Further work will be required to distinguish between these two possibilities.

To our knowledge, this represents the first biochemical evidence for the preferential localisation of a phosphorylated pool of focal adhesion components at the adhesive sites. The selective accumulation of tyrosine-phosphorylated proteins to focal adhesions suggests an important role of the phosphorylated form of these proteins in the formation and maintenance of focal adhesions.

4.3 $[Ca^{2+}]$ REGULATES β1 INTEGRINS FUNCTION AND DISTRIBUTION ON VPMs

The analysis of the mechanisms involved in focal adhesion formation and regulation is complicated by the fact that several events may concomitantly occur during assembly and disassembly of a focal adhesion in the living cell. The possibility to uncouple some of these events would facilitate the analysis of the mechanisms involved. The presence of accessible integrin receptors in an intact lipid bilayer in VPMs makes this an ideal system for the experimental manipulation of integrin function and distribution.

By using VPMs as a "cell-free" system, we found that endogenous β1 integrins rapidly redistribute within VPMs incubated at different $[Ca^{2+}]$s. In particular, high



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Tyrosine-phosphorylated paxillin represents only the 10% of the pool of paxillin present in the cell, and it is selectively accumulated at sites of focal adhesions. At these sites, integrin receptors are clustered and bound to the extracellular ligand from one side, and to intracellular focal adhesion components from the other side.

phosphorylated band immunoprecipitated with FAK antibodies showed an enrichment of the tyrosine-phosphorylated form of FAK in VPM lysates.

FAK and paxillin are two of the best characterised focal adhesion components, and they are tyrosine-phosphorylated during cell adhesion to the extracellular matrix (Burridge *et al.*, 1992; Kornberg *et al.*, 1992). While FAK may regulate the function of focal adhesion components by phosphorylation, paxillin is believed to play a role in the assembly of the focal adhesions, by forming complexes with several components of the adhesive sites, as shown by *in vitro* and *in vivo* studies (Birge *et al.*, 1993; Hildebrand *et al.*, 1995; Sabe *et al.*, 1994; Turner and Miller, 1994; Weng *et al.*, 1993). Although *in vitro* studies have shown that the SH2 domains of Crk, Csk, Src, and Fyn can recognise tyrosine-phosphorylated paxillin (Schaller and Parsons, 1995), the role of paxillin tyrosine-phosphorylation *in vivo* remains to be established. The use of mutants of a major paxillin phosphorylation site (tyrosine 118) *in vivo* (Schaller and Parsons, 1995) indicates that phosphorylation of this site is not required for recruitment to focal adhesions (Bellis *et al.*, 1995), although a number of other phosphorylation sites exist on paxillin that may perform this function. Alternatively, paxillin may become phosphorylated once recruited to the adhesion site by a different mechanism. Further work will be required to distinguish between these two possibilities.

To our knowledge, this represents the first biochemical evidence for the preferential localisation of a phosphorylated pool of focal adhesion components at the adhesive sites. The selective accumulation of tyrosine-phosphorylated proteins to focal adhesions suggests an important role of the phosphorylated form of these proteins in the formation and maintenance of focal adhesions.

4.3 $[Ca^{2+}]$ REGULATES β1 INTEGRINS FUNCTION AND DISTRIBUTION ON VPMs

The analysis of the mechanisms involved in focal adhesion formation and regulation is complicated by the fact that several events may concomitantly occur during assembly and disassembly of a focal adhesion in the living cell. The possibility to uncouple some of these events would facilitate the analysis of the mechanisms involved. The presence of accessible integrin receptors in an intact lipid bilayer in VPMs makes this an ideal system for the experimental manipulation of integrin function and distribution.

By using VPMs as a "cell-free" system, we found that endogenous β1 integrins rapidly redistribute within VPMs incubated at different $[Ca^{2+}]$s. In particular, high

[Ca^{2+}] causes dispersion of integrins out of focal adhesions, while β1 integrins rapidly concentrate along ECM fibrils at low [Ca^{2+}] (see scheme of Fig. 4.2A and B).

To correlate [Ca^{2+}]-induced integrin redistribution with changes in receptor activity we have utilised function-modulating mAbs. It has been recently shown that at least two distinct populations of β1 receptors are present on the surface of CEFs, which may correspond to two different functional states of the receptors (Cruz *et al.*, 1997). By using the function-blocking mAb CSAT (Neff *et al.*, 1982), and the β1-stimulating mAb TASC (Neugebauer and Reichardt, 1991), we have been able to confirm these data on untreated VPMs from CEFs. In addition, the inability of TASC to recognise the receptors on VPMs treated at high [Ca^{2+}] is an indication of the low affinity state of the diffuse β1 receptors, while TASC-positive receptors concentrated along ECM fibrils at low [Ca^{2+}] correspond to high affinity receptors. Interestingly, the stimulatory mAb TASC is able to induce clustering of diffuse receptors along ECM fibrils even in the presence of high [Ca^{2+}]. Since morphological analysis did not reveal any evident [Ca^{2+}]-mediated reorganisation of the ECM, our data suggest that the pattern of distribution of integrins is a consequence of the activation of the receptors induced by low [Ca^{2+}], and depends on the organisation of the available ECM. According to this hypothesis, at low [Ca^{2+}] high affinity (TASC-positive) β1 receptors were diffuse on substrates uniformly coated with purified laminin, where extracellular matrix fibers were not formed.

Intriguingly, VPMs remain spread on the substrate even after incubation for several hours at high [Ca^{2+}], suggesting that adhesion may be mediated by low affinity binding of β1 integrins to the ECM, or by other receptors. Similarly, serum starved Swiss 3T3 cells remain spread and adherent even when no detectable focal contacts are present (Nobes and Hall, 1994), and it has been recently shown that adhesion of serum-starved cells is still RGD-dependent (Barry *et al.*, 1997). The finding that integrin β3 and β5 subunits are present in much lower amounts in CEFs compared to the β1 subunit (Bossy and Reichardt, 1990), suggests that β1 receptors are the major integrin players in VPM adhesion to endogenous ECM. Moreover, analysis of the distribution of the β3 subunit in untreated VPMs showed a diffuse, weak signal, which was not affected by incubation at 37°C at different [Ca^{2+}]s (data not shown). These observations exclude β3 integrins as likely major players in the adhesion of VPMs. On the other hand, given the inability of the function-blocking CSAT mAb to detach VPMs from the substrate (data

shown), the role of other, non-integrin receptors in the adhesion of VPMs to ECM can not be excluded.

An interesting finding of this study is the uncoupling of integrin redistribution from actin stress fibers and focal adhesions under cell-free conditions. Contractility is important for actin stress fibers and focal adhesion formation (Chrzanowska-Wodnicka and Burridge, 1996), and stress fibers are essential for the maintenance of focal adhesions in intact cells (Domnina *et al.*, 1982; Cooper *et al.*, 1987). These and other findings have suggested that the clustering of integrins, while requiring an ECM, is driven from the acto-myosin force generated within the cell (reviewed by Burridge *et al.*, 1997). We find that incubation of VPMs at 37°C leads to a drastic loss of components from the focal contacts of a major fraction of VPMs, indicating that a detectable presence of these proteins at the membrane is not required for integrin redistribution under cell-free conditions. Uncoupling of $[Ca^{2+}]$-induced integrin localisation from the actin cytoskeleton was shown by the fact that disassembly of stress fibers with gelsolin did not affect $[Ca^{2+}]$-mediated receptor redistribution at 37°C, and by the finding that disassembly of stress fibers by gelsolin at 0°C did not perturb receptor localisation into focal contact areas. Our results suggest that mechanisms increasing integrin binding to the ligand are also able to cluster integrins along ECM fibrils, without need of actin-myosin tension. In this direction, Pavalko and Burridge (1991) have shown that disassembly of stress fibers by microinjection of α-actinin fragments into cells only partially affected focal adhesions. On the other hand, we find that reversion of high $[Ca^{2+}]$-induced diffusion of integrins by low $[Ca^{2+}]$ leads to recruitment of the receptors in elongated ECM fibrillar structures which are clearly distinguishable from the focal adhesions found in control VPMs (compare, for example, Figure 3.37A with 3.37C). As depicted in Fig. 3.32, in intact CEFs and in untreated VPMs, β1 integrins clustered into focal adhesions colocalise with the tip of stress fibers on one face of the membrane, and often with part of an ECM fibril on the other face of the membrane (see for example Fig. 3.32A and B). In contrast, low $[Ca^{2+}]$-induced, elongated integrin clusters evidently colocalise with ECM fibrils (see Fig. 3.32E and F), while ultrastructural analysis shows that colocalisation of β1 receptors with stress fibers is not evident under these conditions. These findings suggest that coupling between actin stress fibers and ligand-driven localization of high affinity receptors is required for the recruitment of β1 integrins into focal adhesions of intact cells.

The mechanisms by which divalent cations modulate integrin activity in our system requires further investigations. In particular, from our analysis we can not

conclude whether the effects induced by Ca^{2+} are due to the action of this ion extra- and/or intracellularly. The finding that redistribution in the presence of low $[Ca^{2+}]$ requires the presence of the cytoplasmic domain of the β1 subunit, together with the finding that integrin distribution can not be altered by changing the $[Ca^{2+}]$ in the medium of intact CEFs (not shown), suggests that $[Ca^{2+}]$ could act on the intracellular portion of the membrane, but still an action of $[Ca^{2+}]$ extracellularly could not be excluded. Both possibilities will be discussed below.

1) Extracellular action of Ca^{2+} on β1 integrins function and distribution.

β1 integrins are known to be regulated by extracellular divalent cations, as indicated by studies by using either solubilised receptors or cell binding assays with intact cells (Humphries, 1996). Both integrin α and β subunits contain binding sites for divalent cations in their extracellular portions, which can positively (Mg^{2+} or Mn^{2+}) or negatively (Ca^{2+}) affect integrin-ligand affinity (Tuckwell *et al.*, 1992). Modulation of integrin-mediated cell adhesion to ligands by these cations suggests that the ratio between Mg^{2+} and Ca^{2+} is involved in the regulation of integrin function, which may in turn influence cell behaviour (Grzesiak *et al.*, 1992). According to this model, we found that β1 receptors redistribute on the VPM at different $[Ca^{2+}]$s, in the presence of 2.5 mM $[Mg^{2+}]$. Hypothesising an extracellular action of Ca^{2+}, the requirement of the cytoplasmic tail of β1 for the low $[Ca^{2+}]$-mediated effect on receptor redistribution could be interpreted by an alteration of the conformational state of the deleted receptors, which makes them inactivable by decreasing the $[Ca^{2+}]$. The fact that the mutated receptor could still redistribute upon mAbs stimulation suggests that low $[Ca^{2+}]$ and mAbs mediate receptor redistribution through different mechanisms.

While the diffuse, low affinity β1 integrins could be concentrated along ECM fibrils by lowering the $[Ca^{2+}]$ in the buffer, low $[Ca^{2+}]$ somehow locks the receptors in the high affinity state (see scheme of Fig. 4.2A and B). Diffusion of the receptors could only be achieved by incubation of VPMs with the function-blocking mAb CSAT. One possible explanation for this observation comes from studies pointing at the interplay between distinct cation binding sites in the regulation of ligand binding (Smith *et al.*, 1994; Hu *et al.*, 1996). Mould *et al.* (1995) found that while high $[Ca^{2+}]$ can displace Mg^{2+} from the integrin, low $[Ca^{2+}]$ greatly increased the apparent affinity of Mg^{2+} for its binding site, suggesting the existence of a distinct high affinity Ca^{2+}-binding site. This may lead to more efficient ligand binding by the receptor, that may not be reversed by subsequently increasing the $[Ca^{2+}]$.

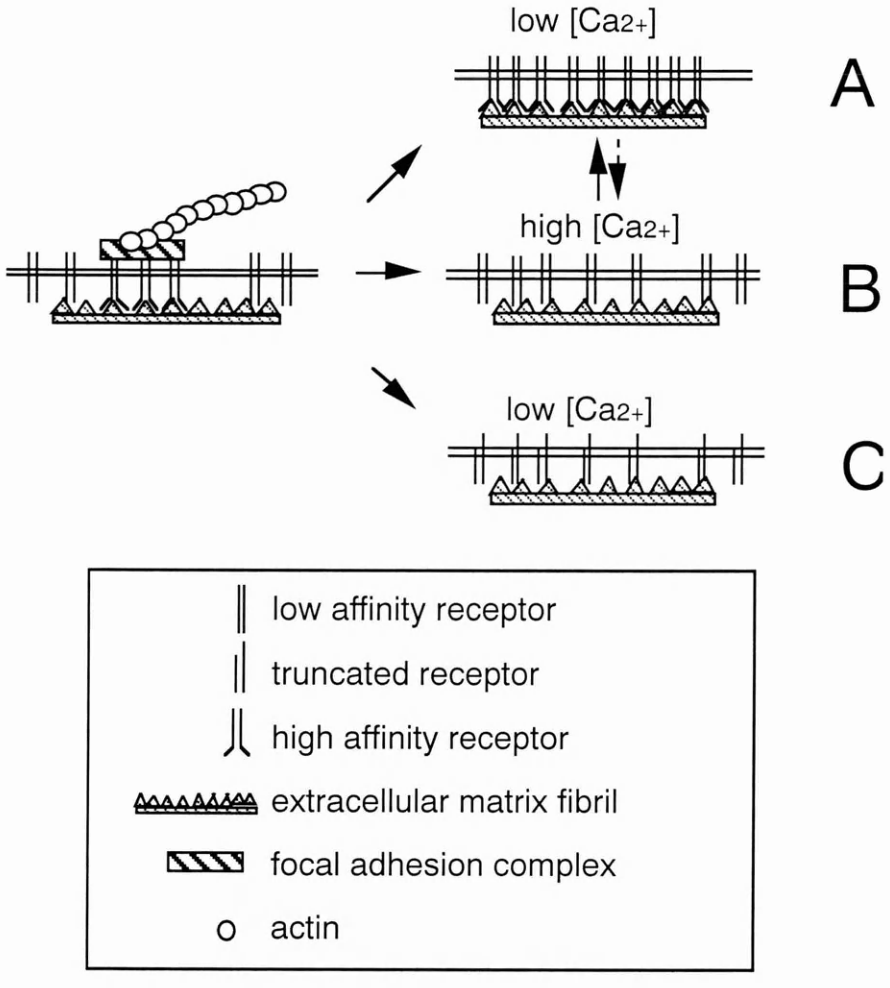

Figure 4.2. Schematic summary of the effects of [Ca^{2+}] on receptor distribution and function.

On the ventral surface of intact cells and in untreated VPMs, β1 integrins are found both concentrated in focal adhesions (high affinity receptors), and diffuse (low affinity receptors). In focal adhesions, integrins colocalise with focal adhesion components, and with the tip of actin stress fibers, while they only partially colocalise with ECM fibrils. (A) Low [Ca^{2+}] induces β1 integrin clustering along ECM fibrils, which may be caused by an increase in receptor affinity for the ligands. (B) High [Ca^{2+}] causes diffusion of β1 integrins out of focal adhesions, and this may be due to a decrease in the affinity of the receptors for the ligands. [Ca^{2+}]-induced redistribution of β1 receptors does not require actin stress fibers, nor detectable amounts of focal adhesion proteins. Moreover, high [Ca^{2+}]-induced receptor diffusion can be quasi-reversed by incubation at low [Ca^{2+}] (full arrow), while low [Ca^{2+}]-induced receptor clustering along ECM fibrils can only be affected by incubation in the presence of receptor function-blocking antibodies (dotted arrow). (C) Low [Ca^{2+}] is unable to induce clustering along ECM of integrins with deleted β1 cytoplasmic domain. In this respect, the finding that activating β1 antibodies are able to induce redistribution of the truncated receptors suggests that low [Ca^{2+}] is not sufficient to activate the truncated integrins, and that low [Ca^{2+}]-mediated activation of the wildtype receptor acts via mechanisms requiring the cytoplasmic domain of the β1 subunit.

The observation that integrin distribution can not be altered by changing the $[Ca^{2+}]$ in the medium of intact CEFs (not shown), can be explained by thinking that other factors present in intact cells, and probably lost during VPMs preparation, are responsible for the control of integrin distribution and function *in vivo*. On the other hand, Stuiver *et al.* (1996) have shown that β3 integrin distribution in intact MG-63 osteosarcoma cells can be regulated by extracellular divalent cation, implicating different mechanisms for different receptors and/or cell types.

In favour of a physiological role of extracellular Ca^{2+} on integrin function, it has been found that free $[Ca^{2+}]$ in the extracellular space is not invariable as previously thought, but can be modified during different biological activities that involve integrin function, such as platelet aggregation, keratinocytes differentiation in the skin, and even during embryonic development, in blood, cerebrospinal fluid and lung (see Maurer *et al.*, 1996, for a review).

2) Intracellular action of Ca^{2+}.

Integrin activation is often mediated by intracellular signals ("inside-out" signalling) that change the affinity of integrins for their ligands (Faull, *et al.*, 1993; Crowe *et al.*, 1994; O'Toole, *et al.*, 1994). In particular, limiting quantities of intracellular factors may bind to β integrin cytoplasmic domains to modulate ligand binding affinity (Chen *et al.*, 1994; Shattil *et al.*, 1995; Colanus, *et al.*, 1996; Chang *et al.*, 1996). Integrin affinity for the ligand is regulated during important biological events, such as cell migration and mitosis, concomitantly with the intracellular $[Ca^{2+}]$ (Alteraifi and Zhelev, 1997; Anilkumar et al., 1996). Moreover, integrins are known to increase the intracellular $[Ca^{2+}]$ in many cell types (Ingber *et al.*, 1990; Hendey and Maxfield, 1993;, Shankar *et al.*, 1993; Zimolo *et al.*, 1994; Sjaadtad *et al.*, 1996), and integrin-mediated Ca^{2+}-signalling has been postulated to participate in a feedback loop that regulates integrin-mediated cell adhesion (Sjaastad et al., 1994). The molecular mechanism of intracellular Ca^{2+} action on integrin function is not known. Although in our experiments 1 mM $[Ca^{2+}]$ gave a more dramatic redistribution of β1 integrins, the effect was evident already at 10 μM (not shown). It could be argued that 10 μM intracellular $[Ca^{2+}]$ is still too high to be considered a physiological condition. In this regard, it is noteworthy that local $[Ca^{2+}]$s nearby the plasma membrane could be higher than the one measured with the usual soluble calcium dyes. Moreover, it has been described that during fibroblasts contraction, transient plasma membrane passages for Ca^{2+} uptake are formed (Lin *et al.*, 1997). Interestingly, Haas and Plow (1996) have demonstrated the formation of a ternary complex between the cytoplasmic domains of

αIIb and β3 and a cation. This finding raises the possibility that Ca^{2+} may act by directly binding to integrins, although an analogous complex with β1 integrins has not been demonstrated yet. More likely, Ca^{2+} could bind to an intracellular factor preserved after VPMs preparation, which in turn would directly or indirectly control integrin function. The irreversibility of the low Ca^{2+}-mediated effect could be explained by a loss of a Ca^{2+}-sensing factor from the membrane after incubation at low Ca^{2+}. A possible candidate for a Ca^{2+}-sensing integrin regulator is calreticulin, a Ca^{2+}-binding protein known to interact with the α chain of integrins in an inducible manner, and proposed to stabilise the high affinity state of integrins (Rojjani *et al.*, 1991; Coppolino *et al.*, 1995; Coppolino *et al.*, 1997). Unfortunately, by using an anti-calreticulin antibody, we were unable to obtain a specific staining for this protein on VPMs (not shown). Another candidate for a Ca^{2+}-binding integrin regulator is suggested by studies on migrating neurophils, where intracellular Ca^{2+} transients control integrin-mediated adhesion, through the action of Ca^{2+}-calmodulin-activated protein phosphatase 2B (calcineurin) (Lawson and Maxfield, 1995). Interestingly, a Ca^{2+}- and integrin-binding protein with homology to calcineurin B has been identified which interacts with the cytoplasmic tail of the integrin αIIb (Naik, *et al.*, 1997). Protein kinase C is known to be activated by Ca^{2+} and to participate in focal adhesion regulation in different cell types (Tang *et al.*, 1993; Woods and Couchman, 1992). Moreover, the receptor for activated protein kinase C (Rack 1) has recently been shown to interact with β1, β2 and β5 cytoplasmic tails (Liliental *et al.*, 1998).

Further work will be necessary for a better understanding of the molecular mechanisms of Ca^{2+}-mediated integrin redistribution and regulation, and the described "cell-free" system would be useful to facilitate the identification of the molecules involved in this process.

4.4 RECONSTITUTION STUDIES ON VPMs

Ex novo reconstitution of adhesive complexes from purified components is made difficult by the fact that focal adhesion assembly occurs at the plasma membrane where clusters of receptors are necessary, and both extracellular ligands and the intracellular milieu may be required. Another major achievement of my work is the reconstitution and modulation of the distribution of a receptor complex in actin-depleted VPMs. Reconstitution of the binding of focal adhesion proteins to permeabilised or partially

for the ligand can be modulated. The use of purified α-actinin, an actin binding protein known to interact *in vitro* with the cytoplasmic tail of integrins (Otey *et al.*, 1990) resulted in decoration of actin stress fibers, confirming published observations (Geiger, 1981). Interactions of integrins with the cytoskeleton via talin (Horwitz *et al ;* 1986) and α-actinin (Otey *et al.*, 1990) are thought to occur as a consequence of integrin-ligand engagement, thus contributing to the formation of focal-adhesion complexes. The novel finding from this study is that after disruption of stress fibers by gelsolin, we can still observe binding of exogenous α-actinin to VPMs in conditions of both high or low affinity of the receptors for the ligands, although a different affinity for the two forms of the receptor can not be excluded. The pattern of distribution of exogenous α-actinin overlaps with that of β1 integrins, and is equally dependent on the $[Ca^{2+}]$ (Fig.4.3 A). The low $[Ca^{2+}]$-induced codistribution of pre-bound, exogenous α-actinin with endogenous β1 receptors strongly indicates that they are part of the same complex (Fig. 4.3A). These results suggest that molecular interactions which are believed to be important for focal adhesion assembly can be reconstituted in a "cell-free" system, and that modulation of the partially reconstituted receptor complex is feasible in this experimental setup. Moreover, the observation that α-actinin can bind to both the active and the inactive form of the receptor suggests a revision of the simple model indicating integrins binding to the cytoskeleton as an event subsequent to receptor activation by the ligand.

Reconstitution experiments with VPMs have also evidenced a special property of focal adhesion complexes in recruiting exogenous actin under conditions in which actin polymerisation is inhibited (diagram of Fig. 4.3B-1 and 4.3B-2). The concentration of cytochalasin D used should indeed block the barbed ends of the actin filaments present (Cooper, 1987). On the other hand, the amount of DNase I used should block all pointed ends and may also bind to the exogenous G-actin. If this is true, exogenous G-actin recruitment to preformed actin filaments should be prevented. Our finding indicates that the actin filament ends are still available at focal adhesions, suggesting that these sites must protect the actin filaments from cytochalasin D and DNase I. One alternative explanation could be that focal adhesions contain proteins which may specifically recruit available G-actin monomers or dimers, by interacting to available sites on the actin molecule. Such a complex may regulate filament growth and may have the ability to nucleate actin polymerisation under appropriate conditions. The finding that the

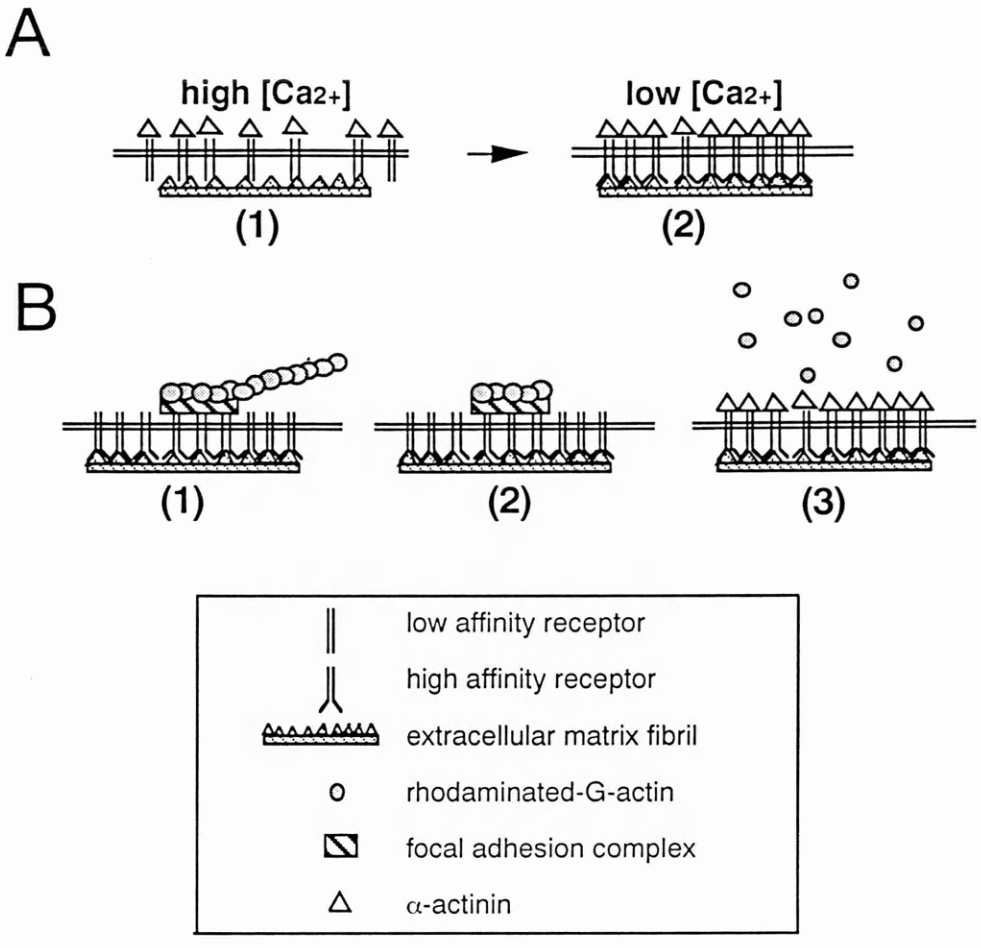

Figure 4.3. Schematic summary of the reconstitution experiments on VPMs.

(A) Exogenous α-actinin bound to actin-depleted VPMs colocalises with β1 integrins at high [Ca^{2+}] (1), and redistributes with integrins along ECM fibrils at low [Ca^{2+}] (2).
(B) Exogenous actin added to VPMs polymerises along stress fibers (1). Under conditions which prevent actin polymerisation (in the presence of cytochalasin D and DNase I), recruitment along stress fibers is prevented, but exogenous actin is still specifically recruited to focal adhesions (2). In reconstitution studies, a complex between activated integrins and α-actinin is not sufficient to recruit exogenous actin on VPMs when actin polymerisation is prevented (3).

reconstituted integrin-α-actinin complex fails to support recruitment of exogenous G-actin to the cell membrane (diagram Fig. 4.3B-3) is not surprising, considering that α-actinin is a bundling protein, able to interact with F-actin. On the other hand, this results would support the hypothesis that exogenous actin is recruited as monomers in the focal adhesions of VPMs, and that focal adhesions represent preferential actin nucleation sites on the membrane.

4.5 FINAL CONCLUSIONS AND PERSPECTIVES

Several conclusions can be drawn from the characterisation and use of VPMs as a "cell-free" system to study integrin function and focal adhesions regulation:

1) In VPMs, Rac 1 and ezrin localise on the actin cytoskeleton and in focal adhesions, respectively.

2) Focal adhesions represent sites for specific accumulation of the tyrosine-phosphorylated form of focal adhesion components, such as paxillin.

3) $[Ca^{2+}]$ affects β1 integrin localisation in a cytoskeleton-independent manner, and a correlation exists between the pattern of distribution of the receptors and their activation state.

4) The relocalisation of the receptors in the presence of low $[Ca^{2+}]$ requires the cytoplasmic portion of the integrin β1 subunit.

5) The use of the "cell-free" system has allowed the partial reconstitution of a $[Ca^{2+}]$-modulated receptor complex in F-actin-depleted membranes.

6) Focal adhesions represent a site for specific recruitment of exogenous actin under conditions that prevent actin polymerisation along stress fibers.

Altogether, these results show that modulation of integrin affinity in the presence of the extracellular ligands is not sufficient to organise focal contacts, although it is sufficient to induce integrin clustering along ECM fibrils. On the other hand, the possibility to reconstitute receptor complexes in a "cell-free" system where integrins are maintained in an intact lidip bilayer, and their function can be modulated, will open the possibility for further reconstitution studies on the functional interactions occurring at focal adhesion sites. Furthermore, the identification of experimental conditions allowing specific recruitment of actin into focal adhesions, together with the localisation on the actin cytoskeleton of proteins known to play a central role in the regulation of the cytoskeleton, can be utilised to further explore the molecular machinery connecting adhesive receptors to actin organisation by utilising the described "cell-free" system.

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